Department of Virology

Haartman Institute

University of Helsinki

Finland

DIAGNOSTICS AND EPIDEMIOLOGY OF HUMAN BOCAVIRUSES

Kalle Kantola

ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in Lecture Hall 2, Haartman Institute, Haartmaninkatu 3, on June 13th, 2014, at 12 noon.

Helsinki 2014

SUPERVISORS: Maria Söderlund-Venermo, PhD, docent

Klaus Hedman MD, PhD, professor

Department of Virology

Haartman Institute

University of Helsinki

Helsinki, Finland

REVIEWERS: Petri Susi, PhD, docent

Department of Virology

Institute of Biomedicine

University of Turku

Turku, Finland

Modra Murovska, MD, PhD

August Kirchenstein Inst. of Microbiology and Virology

Riga Stradins University

Riga, Latvia

OPPONENT: Susanne Modrow, Professor

Institute of Medical Microbiology and Hygiene

University of Regensburg

Regensburg, Germany

The truth may be puzzling. It may take some work to grapple with. It may be counterintuitive. It may contradict deeply held prejudices. It may not be consonant with what we desperately want to be true. But our preferences do not determine what's true. We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth — never there, just closer and closer, always finding vast new oceans of undiscovered possibilities.

CONTENTS

1 ABSTRACT

Parvoviruses are single-stranded DNA viruses that infect both vertebrates and insects. They are among the smallest viruses known with a diameter of only 18-26 nm and a genome of 4-6 kb. Since 2005, six new species of human parvoviruses have been reported, quadrupling the number of known human parvoviruses to eight. Among these are four human bocaviruses (HBoV1-4). HBoV1 was discovered in human respiratory specimens and is likely to cause respiratory disease in young children. In contrast, HBoV2-4 were discovered in human stool specimens and HBoV2 has been tentatively associated with acute gastroenteritis. At the moment there are too little data to evaluate the associations of HBoV3 or HBoV4 with human disease.

To facilitate the diagnostics of HBoV1-4 infections, we developed a multiplex real-time PCR method for their detection in a single reaction (Study I). Differentiation and precise quantitation of the individual bocaviruses can be subsequently achieved with singleplex PCRs that are based on the same primers as the multiplex reaction, or by sequencing. These newly developed PCR methods were shown to be highly specific and sensitive, with detection limits of <10 copies/reaction. The assays were used to assess the prevalence of HBoV1-4 DNA in 250 Finnish adults and children with gastrointestinal symptoms. HBoV2 was detected in four (1.6%) subjects, HBoV3 in one (0.4%) whereas no HBoV1 or HBoV4 DNAs were detected. All four HBoV2-positive children were <2 years of age. In this age group of 20 children, HBoV2 prevalence was as high as 20%. Together with earlier studies, these findings support the conclusion that HBoV2 infections are common among young children but rare among adults.

To expand the tool repertoire in the diagnosis of HBoV infections and to study the immunology of these novel viruses, antibody assays based on HBoV capsid proteins VP2 and VP1 were developed. These proteins are derived from overlapping reading frames and are identical except for an additional ~130 amino acids on the amino terminus of VP1. Immunoblotting showed that, in denatured form, HBoV1 VP2 capsid protein was superior in immunoreactivity to the non-overlapping or "unique" portion of VP1 (VP1u; Study II). This finding was surprising since human parvovirus B19 VP1u is very immunoreactive and useful in the diagnostics of B19. Immunoblot analysis of paired serum samples from children with acute wheezing showed that primary, symptomatic HBoV1

infections frequently result in viremias, elicit B cell immune responses and can be diagnosed using serology. Enzyme immunoassays (EIAs) based on conformational virus-like particles (VLPs) were also developed to refine our understanding of human bocavirus epidemiology (Study III). It was found that HBoV1-4 antibodies are cross-reactive and correction for cross-reactivity is a prerequisite for accurate VLP-based HBoV seroepidemiology.

In Study IV, the newly developed antibody and DNA assays were used to assess the incidences of HBoV1-4 primary infections among constitutionally healthy Finnish children. The incidence of HBoV1-4 viremias and the stability of HBoV1-4 IgG responses were also studied. The study population consisted 109 children of whom serum samples (n=1961) were collected with 3 to 6 month intervals, starting from infancy and up to 13 years of age. All children became seropositive for one or more human bocaviruses by the age of 5 years. The median HBoV1-3 IgG seroconversion ages were 1.7 to 1.9 years. IgG seroconversions for HBoV1-4 were observed in 86%, 53%, 10% and 0% of the children, respectively. Typically HBoV1 IgG levels remained high throughout the follow-up period, whereas HBoV2 IgG levels were markedly lower and characterized by waning and sharp fluctuations. In contrast to acute HBoV1 infections that appear to be mostly viremic, primary HBoV2 infections were found to be rarely associated with viremia or to be short in duration.

HBoV1 IgG seroconversions were rarely detected in children who had HBoV2 IgG and vice versa. In children who nevertheless had IgG towards both viruses, antibody responses to the second infecting virus were typically very weak. This weakness of secondary IgG responses was evident in most of the 9 children who had antibodies for one bocavirus species before showing viremia for another species. Of these nine children, seven did not develop detectable levels of specific antibodies against the second infecting bocavirus. Put together, these results indicate that priming with one bocavirus species may inhibit generation of antibodies toward the novel epitopes of another bocavirus species, a phenomenon known as "original antigenic sin" (OAS). Alternatively, or possibly intertwined with OAS, infection with one bocavirus species may result in cross-protective immunity against other bocavirus species. This weakness or absence of secondary HBoV antibody responses should be taken into consideration when diagnosing HBoV infections serologically.

2 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their roman numerals:

I   Kantola, K., Sadeghi, M., Antikainen, J., Kirveskari, J., Delwart, E., Hedman, K. and Söderlund-Venermo, M. (2010). Real-time quantitative PCR detection of four human bocaviruses. Journal of Clinical Microbiology 48: 4044–4050.

II   Kantola, K., Hedman, L., Allander, T., Jartti, T., Lehtinen, P., Ruuskanen, O., Hedman, K. and Söderlund-Venermo, M. (2008). Serodiagnosis of human bocavirus infection. Clinical Infectious Diseases 46: 540–546.

III   Kantola, K., Hedman, L., Arthur, J., Alibeto, A., Delwart, E., Jartti, T., Ruuskanen, O., Hedman, K. and Söderlund-Venermo, M. (2011). Seroepidemiology of Human Bocaviruses 1-4. Journal of Infectious Diseases 204: 1403–1412.

IV   Kantola, K., Hedman, L., Tanner, L., Simell, V., Mäkinen, M., Partanen, J., Veijola, R., Knip, M., Ilonen, J., Hyöty, H., Toppari, J., Simell, O., Hedman, K. and Söderlund-Venermo, M. Serological responses to Human bocaviruses: new insights from childhood follow-up study. Submitted.

3 ABBREVIATIONS

Abs	absorbance
ADE	antibody dependent enhancement
AEW	acute expiratory wheezing
AGE	acute gastroenteritis
ARD	acute respiratory tract disease
ARTI	acute respiratory tract infection
ALRTI	acute lower respiratory tract infection
AVG	average
BSA	bovine serum albumin
COPD	chronic obstructive pulmonary disease
COV	coefficient of variation
CsCl	cesium chloride
CnMV	canine minute virus
Cq	quantitation cycle [in quantitative polymerase chain reaction]
DENV	dengue virus
DENV1	dengue virus 1 (DENV2, dengue virus 2 etc.)
DNA	deoxyribonucleic acid
DIPP	diabetes prediction and prevention
DOI	digital object identifier; can be translated into a standard web address at dx.doi.org
E. coli	*Escherichia coli*
EIA	enzyme immunoassay
EM	electron microscopy
GE	gastroenteritis
HAI	hemagglutination inhibition
HBoV	human bocavirus
HBoV1	human bocavirus 1 (HBoV2, human bocavirus 2 etc.)
ICTV	International Committee on Taxonomy of Viruses
IgG	immunoglobulin G
IgG_a	analytical immunoglobulin G result obtained after absorption with heterologous HBoV antigen(s)
IgM	immunoglobulin M

IgM$_a$	analytical immunoglobulin M result obtained after absorption with heterologous HBoV antigen(s)
IPTG	isopropyl β-D-1-thiogalactopyranoside
IUPAC	international union of pure and applied chemistry
kb	kilobase
kDa	kilodalton
LD$_{50}$	a dose that is lethal to 50% of the study population
mAb	monoclonal antibody
MCPyV	Merkel cell polyomavirus
MGB	minor groove binder
mRNA	messenger ribonucleic acid
Ni-NTA	nickel-nitrilotriacetic acid
NPA	nasopharyngeal aspirate
NPAFP	non-polio acute flaccid paralysis
NPFS	nasopharyngeal flocked swab
NPS	nasopharyngeal swab
NP1	nuclear phosphoprotein 1
mRNA	messenger ribonucleic acid
NS1	non-structural protein 1
nm	nanometer
nt	nucleotide
OAS	original antigenic sin
OD	optical density
ORF	open reading frame
PARV4	human parvovirus 4
PBS	phosphate buffered saline
PBSP	phosphate buffered saline with 0.05% (v:v) polysorbate 20
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNG	range
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF9	a clonal isolate of Spodoptera frugiperda insect cells
ssDNA	single-stranded DNA

UNG	uracil-N-glycosylase
VLP	virus-like particle
VP1	virus protein 1
VP1u	unique region of virus protein 1
VP2	virus protein 2

4 FOREWORD

Obstetricians and an occasional dog owner recognize the word "parvovirus" and are aware of the serious infections that these pathogens may cause in human fetuses or canine puppies. However, few are aware that there are more than 50 parvovirus species that can infect at least 12 vertebrate species and multiple invertebrate species, ranging from bovines to moths. It is impossible to tell how many exciting new parvoviruses are yet to be found; at least 14 new species have been reported during the last 8 years alone. On average that is almost two new species per year!

Due to the vast size of the virus family, most of the fascinating world of parvoviruses falls outside the scope and limits of this thesis. The literature review therefore focuses almost entirely on the parvovirus genus *Bocavirus*. The interested reader who wishes to explore other aspects of parvoviruses is encouraged to read the excellent book "Parvoviruses", edited by Jonathan Kerr, Marshall Bloom, Susan Cotmore, Michael Linden and Colin Parrish.

The first human bocavirus, HBoV1, was discovered in 2005 and three more species, HBoV2-4, were reported in 2009 and 2010. Relatively little research data has yet accumulated on the latter three viruses. To put the scarce clinical data from humans into context with the known features of other bocaviruses, the first chapter of the literature review investigates the closest pathogenetically characterized relatives of HBoV1-4, the bovine and canine bocaviruses. This also provides unique, tissue-level viewpoints into the pathogenesis of bocavirus infections that would be infeasible in human studies.

The second chapter of the literature review deals specifically with human bocaviruses. The focus is on the DNA-based diagnostics of HBoV infections and their clinical significance. Serological aspects of HBoV diagnostics are addressed in the Results & Discussion section.

The third and the last chapter of the literature review discusses immunological phenomenon known as original antigenic sin, i.e. the propensity of the immune system to utilize immunological memory based on a previous infection when a second slightly different version of a virus (or another antigen) is encountered. Original antigenic sin may have an important role in human

bocavirus immunology and therefore good understanding of the phenomenon may be essential for interpreting the results of this thesis.

   As the last introductory note to the reader, the results section contains a reference to an online supplementary PDF file. The file summarizes 327 graphs of the serological results of Study IV. The contents of the file are not essential for the understanding of the thesis but may provide thought-provoking information to an interested reader. The online location of the file is not provided as a typical Internet address ("URL") but rather as a digital object identifier (DOI), since the former can change over time but the latter is permanent. The DOI code can be translated into a URL at dx.doi.org to gain access to the file, which is hosted by LabArchives Ltd. The company asserts to provide permanent storage of scientific data to scientific journals and individual scientists alike.

5 REVIEW OF THE LITERATURE

5.1 INTRODUCTION TO PARVOVIRUSES

5.1.1 HISTORY

Parvoviruses are among the smallest DNA viruses to infect mammalian cells. The icosahedral capsid has a diameter of only 21.5 [1] to 25.5 nm [2], i.e. roughly 300 times the diameter of a carbon atom, currently only surpassed in minuteness by some circovirus species. Hence the name parvo, originating form the Latin word *parvum* and meaning small.

   The first vertebrate parvovirus was isolated from a rat in 1959 and the first invertebrate parvovirus from a moth in 1964. The mid-1960s also saw the discovery of adeno-associated viruses, a group physically similar to the rodent and invertebrate parvoviruses but dependent upon adenovirus co-infection for their own replication. The name "parvovirus" was introduced to the literature in 1966 and the taxonomic family *Parvoviridae* was established by the International Committee on Taxonomy of Viruses (ICTV) in the 1970's [3].

5.1.2 TAXONOMY & PHYLOGENY

Until the late 90's, ICTV classification of parvoviruses into genera emphasized their biological and structural characteristics [3]. These criteria included factors such as the number of promoters in the virus genome, capability to replicate autonomously without a helper virus and the type of single stranded DNA (sense or antisense) that is packaged in the virion [3]. In 2001, Lukashov and Goudsmith [4] carried out a detailed phylogenetic analysis of all 41 parvovirus genomes that were known at the time. They found discrepancies in the existing ICTV classification of viruses within *Parvovirinae*, e.g. by demonstrating that avian parvoviruses, at the time classified as members of the *Parvovirus* genus, were evolutionarily linked to adeno-associated viruses rather than autonomous parvoviruses. Probably prompted by Lukashov's and Goudsmith's study, ICTV re-evaluated parvovirus taxonomy to better reflect the phylogenetical rather than biological characteristics of the various species. The family is currently

divided into two subfamilies and five genera (Figure 1) and a sixth genus, *Partetravirus*, has been proposed to accommodate the recently discovered PARV4 (also known as human partetravirus; [5]). The *Parvovirinae* subfamily comprises vertebrate-infecting parvoviruses, whereas members of the *Densovirinae* subfamily only infect invertebrates such as certain species of mosquitos, cockroaches and moths [6].

The reader is warned that one of the genera in the subfamily *Parvovirinae,* genus *Parvovirus,* often creates confusion of taxonomical terms, because the English name "parvovirus" is used commonly (and also in this thesis) as an umbrella term to refer to all or any individual *Parvoviridae* species. To clarify this issue, the ICTV *Parvoviridae* Study Group has very recently put forward a proposal[i] to change the genus name to "*Protoparvovirus*". This is part of a large set of proposals by the study group to rationalize and extend the taxonomy of the *Parvoviridae* family. It is therefore likely that parvovirus taxonomy will undergo major changes in the near future.
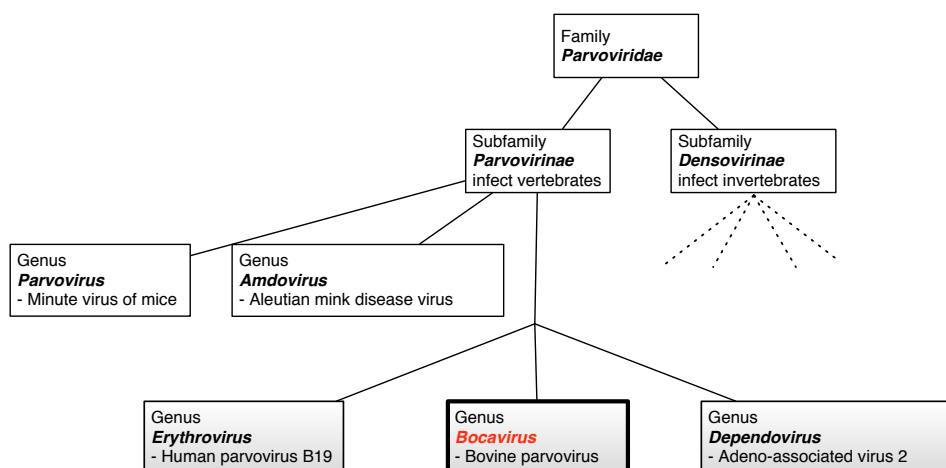
Figure 1. Taxonomy of the Parvoviridae family according to [5]. The type species of each genus in the Parvovirinae family is shown as an example. Genera that include human viruses are shown lowermost and have been shaded with grey. Genera under insect-infecting Densovirinae are not shown.

The focus of this thesis, the recently discovered human bocaviruses, belong putatively to the genus *Bocavirus*. The genus derived its name from the only two present members, <u>bo</u>vine parvovirus and <u>ca</u>nine minute virus, discovered in 1961 [7] and 1970 [8]. At least 8 new putative members of the genus *Bocavirus*

---

[i] The taxonomic proposals have been submitted for peer review. The version currently pending ICTV's internal review can be found with the DOI code 10.6070/H45X26VR.

have been reported between 2005 and 2013 in addition to the four human bocaviruses. They have been tentatively named gorilla bocavirus 1[9], porcine bocavirus[10], porcine boca-like virus[11], canine bocaviruses (number of species uncertain; [12]) and California sea lion bocaviruses 1-4 [13]. The phylogeny of these putative *Bocavirus* species is illustrated in Figure 2, together with representative examples from other *Parvovirinae* genera. The tree shown in Figure 2 has been determined based on the sequence that is most important for the serological aspects of this thesis, the amino acid sequence of the major capsid protein VP2. However, the mentioned set of ICTV *Parvoviridae* Study Group proposals includes a suggestion to use the amino acid sequence of the NS1 protein (described in chapter 5.2.2) as the basis of parvovirus taxonomy. NS1 version of the phylogenetic VP2 tree is included in Supplementary Figure 1. Based on the NS1 phylogeny, the Study Group is proposing the unification of HBoV1 and Gorilla bocavirus 1 under a single species, *Primate bocaparvovirus 1*, whereas HBoV2 and HBoV4 would form another species termed *Primate bocaparvovirus 2*.
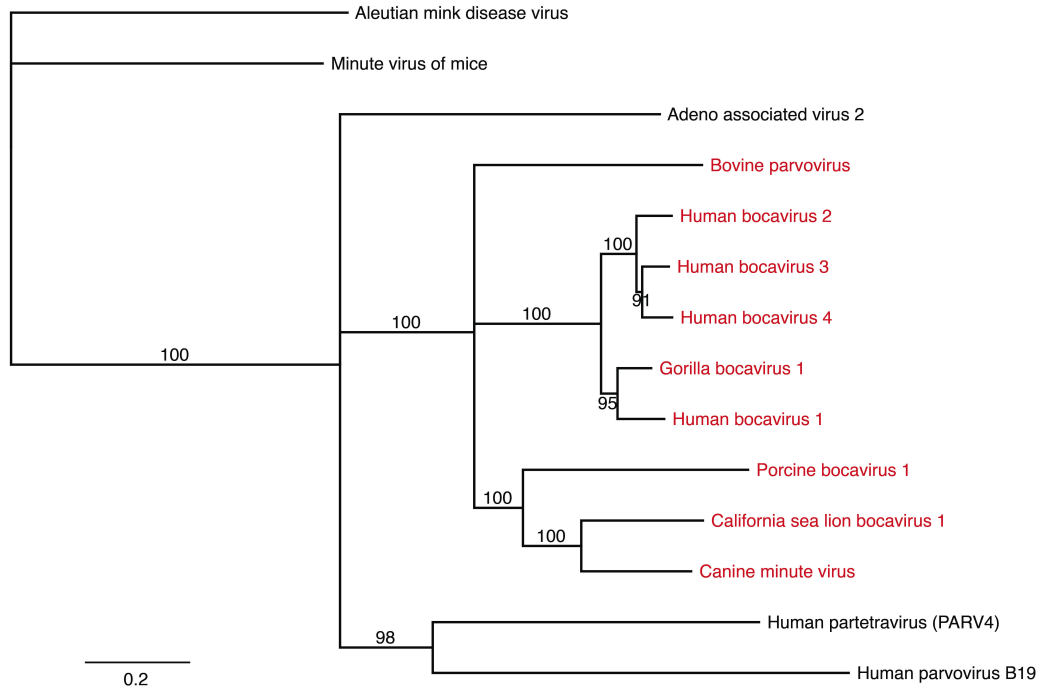
Figure 2. Partial neighbor-joining tree of the Parvovirinae subfamily based on the amino acid sequences of the inferred major capsid protein VP2. Genus Bocavirus is shown in red and has been expanded to show most species within the genus, whereas only the type species are shown from each of the other genera. Sequences were aligned with Geneious 4.8.5 aligner using blosum62 cost matrix with gap open penalty of 12 and gap extension penalty of 3. The tree is derived from 100 bootstrap replicates and bootstrap values ≥70% are shown. The scale bar represents 0.2 average amino acid substitutions per site. GenBank accession number for each sequence is shown in Supplementary Table 1.

As indicated in Figure 2, HBoV1 is phylogenetically closer to gorilla bocavirus 1 than the other human bocaviruses. For instance, the major capsid protein VP2 of HBoV1 has 90% amino acid identity with gorilla bocavirus whereas HBoV1 and HBoV2-4 share ~80% amino acid identity. For comparison, the other human parvoviruses, B19 and PARV4, share only 21-23% VP2 amino acid sequence identity with HBoV1-4 (Table 1).

17

Table 1. Pairwise comparison of VP2 amino acid identities (%) of selected Bocavirus species. GenBank accession numbers for the sequences are listed in Supplementary Table 1.

	HBoV1	HBoV2	HBoV3	HBoV4
HBoV1	-	78	78	80
HBoV2	78	-	89	89
HBoV3	78	89	-	89
HBoV4	80	89	89	-
Bovine parvovirus	47	40	42	45
Canine minute virus	46	44	44	45
Gorilla bocavirus 1	86	80	80	80
B19	23	21	23	21
PARV4	22	23	24	24

5.1.3   PATHOGENICITY OF ANIMAL BOCAVIRUSES

Introduction. Gene sequence analysis enables us to compare the hypothesized pathogenic profile of the novel HBoVs to those of previously characterized parvoviruses. An HBoV profile that was similar to previously characterized members of the genus *bocavirus* could be considered with slightly less skepticism than a completely dissimilar pathogenic profile. Amino acid sequences of the capsid proteins provide a good basis for comparing pathogenicity in this manner since (through interaction with target cell receptors) they are among key determinants of tissue tropism. One could also compare HBoVs to parvoviruses that gain access to host cells via the same receptors. However, HBoV receptors are currently unknown.

Based on the sequences of the VP2 proteins that make up most of the parvovirus capsids, the closest relative to human bocaviruses is gorilla bocavirus 1. However, virtually nothing is currently known about this virus. Porcine bocaviruses and the California sea lion bocaviruses are also largely uncharacterized. However, the two founding members of the genus *Bocavirus*, bovine parvovirus and the canine minute virus (also known as canine parvovirus type-1), have been known since the 60's and the pathological properties of both viruses have been studied in experimental animal models. These animal experiments provide an opportunity to assess the outcomes and pathogenic mechanisms of bocavirus infections in details that would be infeasible with human subjects.

Experimental canine minute virus infections. Carmichael and colleagues inoculated 21 pathogen-free and seronegative, <1-week-old pups oronasally with the canine minute virus and followed histological and clinical course of the infections [14]. Six of the 21 pups (32%) showed clinical signs of illness during the follow-up period. Two of these developed severe illness, one case with fatal outcome. Main symptom among the symptomatic individuals was respiratory distress. Pathological signs included inflammation of the supportive tissue between the alveoli, manifesting as diffuse accumulation of liquid or pus in the alveoli and adjoining ducts (lung consolidation; Figure 3).

Figure 3. Macroscopic appearance of lung from a seriously ill pup, infected experimentally with the canine minute virus (postinoculation day 6). Lung consolidation is apparent in all lobes and visible as dark areas in the image. Reproduced from [14] with permission. Copyright SAGE Publications 1994.

Viral titers in the pups were examined in several tissue types. The median TCID$_{50}$[ii] titer of the lung tissue specimens was the highest (5×10^4), followed in descending order by the median titers of small intestines (3×10^3), lymph nodes (3×10^3), spleens ($<5\times10^1$) and thymi ($<5\times10^1$). All these tissue types were positive in the necropsied pups. The highest lung titers were noted in the two pups that developed the worst symptoms ($\geq 10^6$). The virus was not isolated

---

[ii] TCID50 or "tissue culture infective dose" is a measure of infectious virus titer. It refers to the endpoint titer of the virus-containing specimen required to cause cytopathic effect or lysis in 50% of inoculated tissue culture cells.

from any of the 4 noninfected control pups or from the brain, kidney, liver, heart muscle or bone marrow samples of the infected pups.

Histologic findings in the small intestine were minimal. Only scattered cells in the intestinal glands and villi tested positive for viral antigen by immunofluorescence as opposed to the strong staining of alveolar and bronchial epithelium. Disruption of the normal architecture of the villi was not reported. Authors noted the occurrence of viremia and fecal virus shedding between days 2-8 but did not specify the number of pups testing positive in serum or feces.

In another study, the same authors [15] studied the pathogenicity of the canine minute virus for fetuses by inoculating oronasally 7 seronegative bitches between gestational days 25-35. Five of the 7 showed viremia that, according to virus cultures, lasted 1 to 3 days post-infection but none had clinical illness. All dogs seroconverted to the virus within 2 weeks PI. Three of the 7 bitches lost all fetuses within two weeks from inoculation by embryo resorption and one gave birth to sick pups that all died soon after birth. Three gave birth to healthy pups. Thirteen more fetuses in three additional bitches were inoculated via the amniotic sac between gestational days 35-44. All these fetuses died within 2 weeks from inoculation. Viral titers were measured in amniotic fluid, placenta, lung, heart, thymus and the final portion of the small intestine (ileum). The highest titers among all infected fetuses were observed in the lungs and ileum. As with the older pups, the lung titers of the newborn dogs were consistently at least 10 times higher than those of other measured tissues.

Experimental bovine parvovirus infections. In one of the most detailed studies on experimental bovine parvovirus infections, Durham and Johnson [16] inoculated 15 calves that had been kept in isolation until maternal antibodies against the virus had been reduced to low or non-detectable levels. Six of the calves were inoculated orally, four were not inoculated but were left in contact with the inoculated individuals and five animals were retained as isolated controls. The inoculated calves developed mild to moderate diarrhea, fever and reduced leucocyte counts within two or three days from inoculation. The in-contact controls also developed diarrhea, whereas the uninoculated control calves remained asymptomatic. Microscopic study of the small intestines revealed significant damage to the villi and intestinal glands only 32 hours after infection, as illustrated in Figure 4.
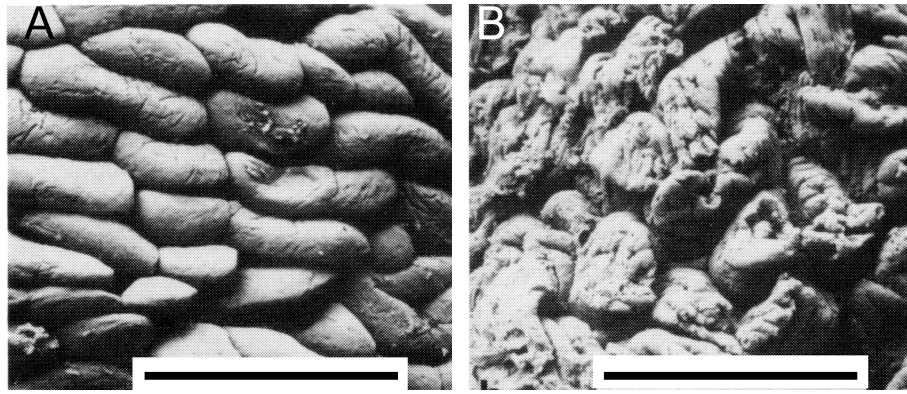
Figure 4. Scanning electron micrographs of the ileal mucosa of two calves; (A) healthy control, (B) 32 hours after infection with the bovine parvovirus. Bars represent 400 μm. Adapted from [16], with permission. Copyright Elsevier 1985.

Inspection of sequential necropsy tissues by immunofluorescence microscopy indicated that tonsillar tissues, intestinal mucosa and to a minor degree mesenteric lymph nodes all became infected with bovine parvovirus within 32 hours from inoculation. Most prominent fluorescence was reported in the glandular epithelia of the small intestine. These tissues remained positive after 3 days, at which point a relatively large number of fluorescent cells were also observed in the thymus. After 5 days from inoculation, only the final section of the small intestine (ileum) and thymus remained positive in immunofluorescence microscopy. Tonsil, the portions of small intestine preceding ileum (duodenum & jejunum), colon, spleen and all tested lymph nodes were negative at this point. No fluorescent cells were seen in liver, lung, kidney, heart or testes at any time point.

In a similar experiment, Storz and colleagues [17] studied bovine parvovirus infection in orally inoculated calves and reported organ distribution and symptoms that were very similar to those described above. In another experimental infection, Spahn and colleagues [18] did not carry out histological analyses but noted that orally inoculated calves developed only diarrhea, while those inoculated intranasally also developed a mild respiratory illness in the form of nasal discharge. In contrast to the mostly asymptomatic canine minute virus infections in pups (see previous subchapter), all four studies reported mild to moderate diarrhea in all inoculated calves.

Principal target cells. It is easy to see why parvoviruses would target the intestinal mucosa. The cells that cover this surface are very short-lived and are continuously replaced by new cells. Parvoviruses are generally believed to

require these kinds of rapidly dividing cells or helper viruses for efficient replication because their minute genome does not carry sufficient genetic information to code for their own replicative enzymes [3,6,19]. Studies also have yet to show that parvoviruses could force the host cell into S-phase of replication.

The most vigorously replicating cells in the small intestine are located in intestinal glands, also known as Crypts of Lieberkühn, which produce the epithelial cells of the villi. The basal portion of the crypt contains multipotent stem cells. After each mitosis, one of the two daughter cells migrates up to the side of the crypt and eventually into the villus while the other remains in the crypt as stem cell [20]. The study by Durham and Johnson supported the notion that bovine parvovirus specifically infect the intestinal glands by showing how the infected cells sequentially moved from the base of the intestinal glands towards the tips of the villi. By disabling the new source of cells, the virus can prevent dying cells on the villi from being replaced. This results in significantly reduced absorption capacity of the intestines, leading to diarrhea and nausea. Even death can result if the intestinal barrier is sufficiently reduced to allow intestinal bacteria to gain access to the bloodstream.

Unlike the rapidly dividing cells of the small intestine, the lung epithelial cells are considered to have a relatively slow renewal rate at least among adults [21]. It is therefore surprising that infection by the minute virus of canines typically results in significantly higher viral titers in the lungs than in other organs. In the already discussed experimental infection of dogs, Carmichael and colleagues [14] demonstrated that the two seriously ill pups had at least 1000 fold higher viral titers in the lung than in the organ with the next highest viral titer, the small intestine.

5.2   HUMAN BOCAVIRUSES

5.2.1   DISCOVERY

The famous Louis Pasteur quote, "chance favors the prepared mind in the field of observation", applies well to the discovery of the first two human parvoviruses. The adeno-associated virus, was observed visually when Atchison and colleagues [22] were inspecting the purity of an adenovirus preparation with an electron microscope. The second human parvovirus, parvovirus B19, was found in a similar manner when researchers used an electron microscope to investigate a serum that gave anomalous results in immunoassays [23]. However, chance was a minor factor in the discovery of the first human bocavirus, HBoV1, by Allander and colleagues [24], and their finding is better described by another aphorism, "success follows excellence".

The discovery was made with a pioneering screening method, also developed by Allander and colleagues [25], which was specifically designed to discover new viruses from human specimens. The method involved the following five phases: 1) enzymatic and mechanical host DNA depletion to enrich viral nucleic acids, 2) amplification of enriched DNA by random PCR, 3) cloning of the PCR products, 4) large-scale sequencing of the clones, and 5) automated editing and database searches of the sequencing results. HBoV1 was found when the screening method was applied on respiratory samples obtained from the patients with a suspected respiratory infection [24]. The virus was identified from DNA clones that did not match any known viruses at the nucleotide level, while simultaneously showing significant similarity to the amino acid sequences of the bovine parvovirus and the canine minute virus.

The concept of random PCR screening was quickly adopted by other researchers, thus widening the systematic exploration of as yet unknown human viruses. This resulted in the discoveries of HBoV2 [26], -3 [26] and -4 [27] in 2009 and 2010. Unlike HBoV1, however, all these three viruses were found in human fecal samples rather than respiratory specimens.

5.2.2   GENOME

The small ~5 kb genome encodes three major separate open reading frames as illustrated in Figure 5. The 5' region encodes the NS1 protein, which appears to be involved in the regulation of HBoV promoter activity [28]. The middle region of the genome encodes the nucleoprotein 1 (NP1), which has an unknown function, and the 3' region encodes the two structural proteins VP1 and VP2. The structural proteins are formed from the same transcript using alternative splicing. As a result, VP1 and VP2 differ only in their N-terminal region and the unique portion of VP1 is known as VP1u.

Studies published so far indicate that human bocaviruses may differ from other parvoviruses with respect to their genome structures and replication mechanisms. Typical parvovirus genomes are considered single-stranded DNA molecules that consist a relatively long single stranded coding sequence bracketed by short imperfect terminal palindromes that fold back onto themselves to form hairpin structures [3], as illustrated in Figure 5. The 3' end of the hairpin functions as a primer to initiate replication of the viral genome from the left-terminal sequence in a so called rolling-hairpin manner [29]. In contrast, recent data indicates that human bocavirus genomes can exist and/or persist as closed circular DNA molecules [30,31]. Figure 5 illustrates the genomic organization of human bocaviruses in the form of an HBoV3 episome that was discovered from an intestinal biopsy [31]. A similar HBoV2 episome was recently reported in the fecal specimen of a diarrheic Chinese child [32].
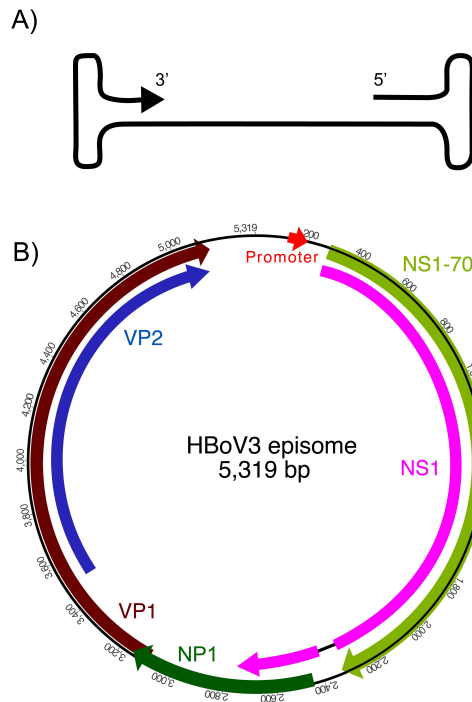
Figure 5. Schematic representation of A) a typical parvovirus genome organization in the hairpin configuration and B) the suggested HBoV episomal configuration with HBoV3 genome as an example. The arrow in image A represents the direction of DNA replication. Image B is based on the GenBank sequence JN086998 and the protein encoding sequences are based on data from [33] and [34] . Encoded proteins are indicated as arrows and the thin line in the NS1 arrow symbolizes an intron gap. See the text for description of each encoded protein.

Sequence analysis of HBoV genome sequences has indicated that their evolution has involved interspecies recombination events. Kapoor and colleagues [27] compared the regional sequence similarities of human bocaviruses and found that the NS1 and NP1 genes of HBoV3 cluster with those of HBoV1 whereas at the other side of the genome, VP1/2 genes cluster with those of HBoV2. HBoV4, on the other hand, is more similar to HBoV2 in NS1/NP1 but with HBoV3 in VP1/2. In one possible chain of events, HBoV3 may have originated from a recombination event between HBoV1 and HBoV2, whereas HBoV4 may have originated from recombination between HBoV3 and HBoV2.

5.2.3 STRUCTURE

The human bocavirus genome is protected by a non-enveloped capsid that, based on native HBoV1 particles, has a diameter of approximately 28 nm [35]. The absence of a lipid envelope and the simple overall structure make parvovirus capsids exceptionally resilient to environmental challenges. Bovine parvovirus particles can withstand 100°C dry heat for 30 minutes with one log reduction in infectivity, whereas typical pasteurization protocol for human serum albumin (3 h at 60 degrees with moist heat) would reduce bovine parvovirus infectivity by three logs [36].

Similar to other parvoviruses, HBoV1 [37] and HBoV2-4 (B. Gurda, personal communication) capsids are icosahedral and consist of 60 copies of structural proteins. Based on studies on human parvovirus B19, VP2 units make up ~95% of the capsid in native particles [38]. The VP2 protein has highly conserved secondary structure core elements that are shared even between parvovirus genera while the variable surface loops confer the host-specific tropism and the diverse antigenic properties [37]. The remaining ~5% of the capsid structure consists of minor proteins, the type of which depends on the virus species. All contain low numbers of VP1 molecules but some species, for example minute virus of mice and porcine parvovirus, also contain VP3, which is formed from VP2 by proteolytic cleavage [3]. Some HBoV1 VP2 molecules also appear to be cleaved to form VP3 [35].

The VP2 monomers of several (and possibly all) parvovirus species can self-assemble into virus-like particles (VLPs) in the absence of other proteins. The production of recombinant VP2 VLPs in insect cells has been demonstrated e.g. for parvovirus B19 [39,40], canine parvovirus type 2 [41], feline panleukopenia virus [41], Aleutian mink disease parvovirus [42] and indeed for HBoV1-4 as discussed in the results section of this thesis. All the referenced studies have reported that the recombinant VP2 capsids appear to be similar to native capsids in appearance and, when measured, in buoyant density. The VP2 capsids have also been successfully used in the serodiagnosis of parvovirus infections (e.g. [43-46]), indicating that also the antigenic properties of the artificial capsids resemble those of the native capsids.

The ability of VP2 monomers to self-assemble into VLPs does not mean that VP1 is dispensable for productive infection. A study on the minute virus of mice (MVM) first demonstrated that virus that lacks VP1 binds to cells

as efficiently as wild-type virus but fails to initiate a productive infection. The requirement for VP1 is thought to be important for endosomal escape [47,48] and nuclear targeting [49]. In the case of parvovirus B19, it appears to be also essential for efficient internalization of the virus [50].

5.2.4 HBoV1 EPIDEMIOLOGY

Respiratory samples. The discovery of HBoV1 in children with respiratory disease rapidly prompted a large number of confirmatory studies assessing the presence of the virus in respiratory specimens. Since these are routinely taken from symptomatic individuals but only rarely from asymptomatic individuals, most studies have focused on HBoV1 prevalence among the former. Results from studies assessing the incidence of HBoV1 DNA in the respiratory samples of patients with respiratory disease are summarized in Table 2. The list of studies was adapted from a 2012 systematic review by Jartti and colleagues [51]. Some studies were excluded based on criteria that are detailed in the table's footnote. It is evident from the tables that HBoV1 infections occur commonly around the globe and are mainly detected in young children aged 6-24 months. In contrast, detection of HBoV1 in the respiratory specimens of immunocompetent adults is rare. Data are scarce on the occurrence of HBoV1 infection among the elderly.

Table 2. Age-stratified[a] HBoV1 DNA prevalences in patients with respiratory disease as measured by PCR from respiratory specimens

Study		Age (years)	Country	% positive	n	Symptoms
[52]	NPS	<1	Thailand	10	67	pneumonia
[53]	NPA	median 0.7 range 0.1 - 4.9	Korea	0	212	ALRTI
[54]	NPA	median 1.2 range 0.1 - 5.8	Korea	8	336	ALRTI
[55]	NPA	median 1.3	Canada	14	225	ARTI
[56]	NPA	median 1.6 range 0.3-15	Finland	19	259	AEW
[57]	nasal swab	<2	USA	33[b]	106	"respiratory illness"
[52]	NPS	1-4	Thailand	12	302	pneumonia
[58]	NPA	mean 3.4 y	Germany	10	835	ARTI
[59]	NPS	<5	Vietnam	2	958	ARTI
[60]	NPA	<5	France	4	589	ARTI
[61]	mixed[c]	<6	Canada	3	290	ARTI
[61]	mixed[c]	6-15	Canada	2	149	ARTI
[52]	NPS	5-19	Thailand	3	132	pneumonia
[52]	NPS	20-49	Thailand	1	213	pneumonia
[61]	mixed[c]	16-50	Canada	1	444	ARTI
[55]	NPA	>40 y	Canada	1	126	COPD
[52]	NPS	50-64	Thailand	1	149	pneumonia
[61]	mixed[c]	>50	Canada	0.3	149	ARTI
[52]	NPA	>65	Thailand	1	305	pneumonia

ALRTI, acute lower respiratory tract infection; ARTI, acute respiratory tract infection; AEW, acute expiratory wheezing; COPD, chronic obstructive pulmonary disease; n, number of subjects; NPS, nasopharyngeal swab; NPA, nasopharyngeal aspirate

[a]The studies have been arranged by age and studies with multiple age groups [52,61] have been split to separate rows with one age group per row.

[b]Multiple samples were obtained from each individual, increasing the likelihood of virus detection. Other studies analyzed one respiratory sample per study subject.

[c]Specimen types analyzed included throat swabs, nasopharyngeal swabs, nasopharyngeal aspirates and auger suctions.

Note: The list of studies is based on a previous systematic review [51]. Studies were excluded from the table if detection was based on a novel PCR method and the results were not verified by sequencing or by another independent assay [62-65]. Also studies with ambiguous descriptions of ages [66] or symptoms [67,68] were excluded.

Several studies have demonstrated the detection of HBoV1 DNA in the respiratory samples of completely asymptomatic individuals and individuals with non-respiratory symptoms. However, before inspecting these data and comparing it to data on symptomatic individuals, there are several important factors that need to be considered:

1) PCR has been used only in a few studies to analyze respiratory samples of truly asymptomatic patients. Several studies have designated subjects as "control patients" or "asymptomatic control patients", although they may have experienced other than respiratory symptoms [52], or taken part in elective surgery [55,69,70]. A frequently performed elective surgery is tonsillectomy, which may correlate positively with HBoV1 infection by direct causal relationship or enhancement of HBoV replication e.g. by recruitment of immune cells permissive for HBoV1 infection (discussed in [71]).

2) Presence of HBoV1 DNA in respiratory samples does not necessarily indicate an acute infection, and by extension, does not by itself demonstrate a subclinical infection in an asymptomatic individual. This has been shown in several studies on immunocompetent children, documenting extended or intermittent HBoV persistence in the respiratory tract. For instance, a Danish prospective birth cohort study found that 25% of 228 young immunocompetent children remained PCR positive at least 2 months after the appearance of HBoV1 DNA in respiratory specimens [65], whereas a Finnish study of otitis-prone children found that 11% of 152 children were HBoV1 DNA positive after 3 months and one child after 6 months [72]. At least two other studies [57,73] have also reported the prolonged or intermittent presence of HBoV1 DNA in respiratory specimens of immunocompetent children.

3) Studies on completely asymptomatic individuals are scarce and the numbers of participants in these few studies are typically small because respiratory samples are not routinely collected from asymptomatic individuals. The small number of individuals increases the likelihood of all subjects showing a negative result.

4) The procedure commonly used for respiratory specimen collection from symptomatic individuals is a painful and unpleasant nasopharyngeal swab or

aspiration (Figure 6), whereas truly asymptomatic individuals have been almost exclusively sampled with significantly less invasive nasal swabs. This difference in sampling procedures would favor the detection of HBoV1 DNA in symptomatic individuals if HBoV1 (or its DNA) preferentially persisted in the nasopharynx or lower parts of the respiratory tracts. Swabs also provide significantly lower volumes of mucous sample material for DNA extraction than aspirates, potentially reducing the likelihood of HBoV1 DNA detection. This difference in detection sensitivities has been previously demonstrated with other viruses. Chan and colleagues [74] measured RSV and influenza viral load in nasopharyngeal aspirates and nasopharyngeal flocked swabs (NPFS) obtained in parallel from 196 hospitalized children with acute respiratory infection and found that, on average, NPA samples yielded 9 times higher RSV and 33 times higher influenza A DNA levels than NPFS samples. Also, Heikkinen and colleagues [75] found that 97% of patients with RSV tested positive in NPA by PCR as opposed to 76% in swab samples (RSV positivity was based on total viral findings by either method). Differences in detection sensitivities between these two methods have not been determined for HBoV, but they are likely to be significant in patients with low viral loads. On the other hand, the increased likelihood of HBoV1 DNA detection in the nasopharyngeal samples of symptomatic patients could be counterbalanced by the greater exposure of the nasal swab sampling area to exogenous ("contaminating") sources of HBoV1 DNA.
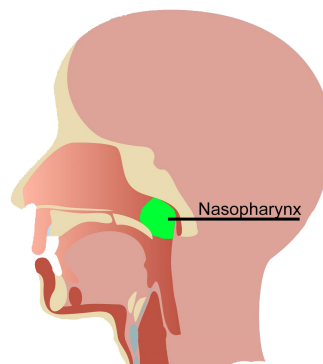
Figure 6. Nasopharyngeal swabs and aspirates are sampled from the uppermost part of the throat, i.e. the nasopharynx, by passing the sampling instrument through a nostril. Image copyright was licensed from 123RF Ltd (Hong Kong, China).

Keeping the above issues in mind, Table 3 lists studies that have assessed the prevalence of HBoV1 in patients without any symptoms or without symptoms

indicative of a respiratory infection. The list of studies has again been adapted from the systematic review of Jartti and colleagues [51] and complemented with another study [76].

Table 3. Age-stratified[a] HBoV1 DNA prevalences in the respiratory samples or stool samples of patients without respiratory or gastrointestinal disease

Study	Sample type	Enrollment criteria	Age (years)	Country	% positive	n
[65]	nasal swab	asymptomatic	<1	Denmark	9	152
[57]	nasal swab	asymptomatic	<2	USA	44	45
[68]	nasal wash	asymptomatic	<2	USA	0	96
[77]	saliva	asymptomatic	2-4	USA	9	56
[70]	NPA	elective surgery	AVG 3.6 RNG not specified	Norway	17	162
[52]	NPS	outpatient clinic, no ARD	<4	Thailand	5	85
[69]	NPA	trauma, elective surgery, well-child visit	<5	France	0	68
[76]	NPS	asymptomatic	<5	China	3	195
[55]	NPA	elective surgery	"children"	Canada	43[b]	100
[78]	NPA	elective surgery, food challenge	"children"	Spain	5	116
[52]	NPS	outpatient clinic, no ARD	5-19	Thailand	0	69
[52]	NPS	outpatient clinic, no ARD	20-49	Thailand	0	54
[52]	NPS	outpatient clinic, no ARD	>50	Thailand	1	72
[79]	stool	asymptomatic	<5	Korea	4	115
[26]	stool	asymptomatic	median 1.8 RNG 0.1-17	Australia	6	186

NPS, nasopharyngeal swab; NPA, nasopharyngeal aspirate; ARD, acute respiratory disease; AVG, average; RNG, range.

[a]The studies have been arranged by the age of the patients and a study with multiple age groups [52] was split to separate rows with one age group per row.

[b]These children were hospitalized for a surgery (mostly ear, nose or throat surgeries) but did not have respiratory symptoms. Results were verified by two independent quantitative PCRs as well as sequencing.

Note: a study with very small number of subjects (<50; [80]) was excluded from the table.

Interestingly, the two highest HBoV1 DNA prevalences ever published have been among individuals without respiratory symptoms. Martin and colleagues [57] performed HBoV testing on nasal swabs in a prospective, longitudinal study of respiratory illness in 119 children who attended daycare, and documented HBoV in 20 of 45 (44%) of asymptomatic enrollment samples.

Longtin and colleagues tested nasopharyngeal aspirates and found HBoV DNA in 43 of 100 (43%) of children undergoing elective surgery (mostly ear, nose and throat surgeries) without concomitant respiratory symptoms or fever at admission [55].

Because of the complicating factors discussed above, it is impossible to assess potential correlation, let alone causal relationship, between HBoV1 infection and respiratory symptoms by comparing Tables 2 and 3. Chapter 5.2.6 addresses correlations and causalities by looking at individual studies in more detail. However, these two tables give us rough approximations of the prevalences of HBoV1 DNA in symptomatic and asymptomatic individuals and show us that the mere presence of HBoV1 DNA in respiratory specimens is not a reliable indicator of a symptomatic respiratory infection. A significantly better sign of an acute infection is likely to be the presence of a high viral load in respiratory specimens or the presence of viral DNA in blood (i.e. viremia). Allander and colleagues found that 88% of children with more than 10^4 copies/ml of HBoV1 DNA in NPA had viremia as opposed to only 11% of children in whom the copy number was <10^4 copies/ml of starting sample [56]. The significance of viremia as a marker of an acute HBoV1 infection is discussed in more detail in the results section of this thesis (chapter 8.6).

Non-respiratory samples. HBoV1 does not appear to show the same type of tissue persistence that characterizes human parvovirus B19. After primary infection, the B19 DNA may remain detectable in the tissues of both symptomatic and asymptomatic subjects for several decades. Persistence of B19 DNA has been reported in synovial membranes (i.e the tissue covering the inside surface of most joint cavities), skin and tonsils with detection frequencies of 16-50% [81,82]. B19 DNA is also frequently found in heart tissue, with one study reporting a DNA prevalence of 85% in 100 patients who underwent open-heart surgery [83]. In contrast, no evidence of HBoV1 DNA persistence has been found in the tonsils of adults or in the skin or synovial tissues of any age group [84]. Only tonsils of young children aged <8 years tested positive for HBoV1 [84] and none the tonsils, synovial membranes or skin tested positive for HBoV2-4 regardless of age. Of the 100 heart patients who were mostly positive for B19 DNA [83], only 5% tested positive for HBoV1 DNA.

Schildren and colleagues [85] tested tissue specimens from lung and colorectal cancers for HBoV1 DNA and found 18% of the lung tumors and 21

of the colorectal tumors positive for the virus. HBoV1 DNA has also been found in stool and urine. Pozo and colleagues analyzed urine samples from six hospitalized LRTI patients and found HBoV1 DNA in two of the children [86], which appears to be the only published study on the subject. The virus DNA has also been found in stools of children with gastrointestinal or respiratory illness. Among these types of children aged <6 years, the range of DNA detection has been 0.5-9% in major studies with at least 300 patients [79,87-91]. Average detection frequency appears to be ~4%. The presence of HBoV1 DNA in stool and urine may well be indicative of swallowed virus or virus filtrated from the blood in the kidneys, rather than active shedding in either secretory pathway. Passive spread of HBoV1 from the respiratory to the gastrointestinal tract is supported by the consistently low levels of viral DNA in stool [92].

HBoV1 DNA detection in the stools of adults is rare. The most comprehensive study on the subject analyzed ~2300 samples from individuals with gastrointestinal disease and ~2100 asymptomatic controls [93]. The ages of the patients and controls ranged from <1 to >70 years with relatively even age distribution in different age subgroups (1-4 y, 5-9 y, 10-19 y, 20-29 y etc). None of the adults tested positive for HBoV1 DNA in contrast to 2% of children aged <4 years.

The ability of parvovirus B19 and the canine minute virus (an animal bocavirus; chapter 5.1.3) to cause intrauterine infection and abortion has prompted research into the possible role of HBoV1 in fetal disease. However, no HBoV1 DNA was found in 87 amniotic fluid specimens from fetuses with hydrops, anemia or isolated effusions [94]. Another study examined fetal tissues (placenta, heart and liver) from 120 miscarriages and 169 intrauterine fetal deaths and found that no HBoV1 DNA [95].

5.2.5   HBoV2-4 EPIDEMIOLOGY

Respiratory samples. Human bocaviruses show significant similarity on amino acid and nucleotide level but appear to differ significantly in tissue tropism. As discussed, HBoV1 is seen mainly as a respiratory virus and the presence of HBoV1 DNA in stool specimens may reflect swallowing rather than gastrointestinal shedding. In contrast, HBoV2-4 are considered enteric viruses because they are mainly detected in stool and only rarely in respiratory specimens. This is reflected by a comprehensive study by Chieochansin and

colleagues [67], involving ~6200 respiratory samples from Scotland and ~400 from India. The ages and symptoms of these patients were not specified but the sample type (mainly NPA) indicates respiratory symptoms. None of the respiratory specimens tested positive for HBoV2 whereas HBoV1 was found in 3% of the Scottish and 14% of the Indian samples. However, the Scottish samples were pooled from 10 individual samples, inevitably resulting in reduced DNA detection sensitivity.

Later studies have demonstrated that HBoV2-4 are not entirely absent from respiratory specimens. Chinese [96], Korean [53] and Japanese [97] studies have respectively reported HBoV2 in 4%, 2% and 0.5% of (non-pooled) respiratory specimens from children with respiratory tract disease. Of these three, the Japanese study was the only one also to test the samples for HBoV3 and -4 DNA. Detection rates for both viruses were ~0.5%. In Finland, Risku and colleagues screened ~1500 nasal swabs from children with acute respiratory tract disease and found HBoV1-4 respectively in 4.5%, 0.2%, 0% and 0% of the samples (M. Risku, submitted). Thus, the incidence of HBoV2-4 in respiratory samples is low in comparison to HBoV1. None of these studies assessed the quantity of HBoV2-4 DNA in the respiratory specimens, since all were based on qualitative PCRs. However, the HBoV2 loads in respiratory specimens are likely to be low considering their complete absence in the aforementioned >6000 pooled English respiratory samples [67].

In the absence of quantitative data showing moderate or high respiratory DNA loads, the mere presence of HBoV2-4 DNA could indicate contamination of respiratory airways by viral DNA from fecal material rather than active shedding. Future studies should address this possibility by testing for HBoV2-4 mRNA rather than DNA as successfully shown for HBoV1 [98].

Stool samples. It is difficult to summarize the age-related incidence of HBoV2 DNA in stool samples in any great detail because most studies have focused on samples from wide age groups and/or provided inadequate information about the age distribution of the study subjects. Table 4 summarizes results from studies that have assessed the occurrence of HBoV2 in symptomatic and asymptomatic individuals.

Table 4. Age-stratified[a] HBoV2 prevalences in the stool of symptomatic and asymptomatic individuals

Study	age (years)	Country	% positive	n	Symptoms
[99]	median 1.5 range 0-16	Korea	4	358	GE
[100]	mean 1.7 range 0-1.7	China	20	632	GE
[26]	median 1.7 range 0-18	Australia	17	186	GE
[27]	median ND range 0-15	Nigeria Tunisia	23	192	NPAFP
[101]	"children"	China	25	345	GE
[27]	"adults"	USA	4	87	GE
[27]	"adults"	Nepal	4	96	GE
[102]	median ND range 0-15	Finland	3	878	GE
[100]	mean 0.9 range 0-1.7	China	12	162	healthy
[26]	median 1.8 range 0-17	Australia	8	186	healthy
[27]	median ND range 0-15	Tunisia	24	96	healthy
[27]	"adults"	USA	0	74	healthy
[27]	"adults"	Nepal	4	96	healthy

ND, not disclosed; GE, gastroenteritis; NPAFP, non-polio acute flaccid paralysis.

[a]The studies have been arranged by age and studies with multiple age groups have been split to separate rows with one age group per row.

Note: studies that had very wide age groups and did not report average or median ages [103,104] were excluded from the table. Results from the study by Risku and colleagues [102] were partially excluded due to very few individuals (<50) in some patient groups. A wavy line separates results from symptomatic and asymptomatic subjects.

HBoV2 prevalence appears to be relatively high among young children and low in adults based on the few studies that do have reasonably narrow age groups. This resembles the occurrence of HBoV1 in respiratory samples. Also reminiscent of HBoV1 is the occurrence of HBoV2 DNA in the stool of asymptomatic children. In fact, the second highest reported HBoV2 prevalence

(24%; highest 25% [101]) has been reported in the stool of 96 asymptomatic Tunisian children [27].

HBoV3 DNA occurs less frequently in stool than HBoV2 DNA but may exceed the occurrence of HBoV1 DNA. A Finnish study of ~900 children with gastroenteritis found HBoV1-4 DNA with respective frequencies of 6%, 3% and 1% [102]. An even more comprehensive English study of ~2000 gastroenteritis patients found HBoV1-3 DNA with respective frequencies of 3%, 22% and 7% [93].

HBoV4 DNA detection appears to be rare in stool specimens in comparison to HBoV1-3, with only two studies reporting the presence of any HBoV4 positive results; one study found the virus in 2% of Tunisian children with acute flaccid paralysis (2%; [27]) and the other in 0.5% of Thai children with gastroenteritis. Three other studies, however, did not find any HBoV4 DNA in stool [102,105,106].

5.2.6 HBoV1-4 CLINICAL SIGNIFICANCE

Criteria for establishing causality. Koch's postulates are three widely cited criteria designed to assess a causal relationship between a causative microbe and a disease. Different translations and interpretations of the original criteria exist, but according to Fredericks and Relman [107] they are:

1. The pathogen occurs in every case of the disease in question and under circumstances, which can account for the pathological changes and clinical course of the disease
2. The pathogen occurs in no other disease as a fortuitous and nonpathogenic parasite
3. After being fully isolated from the body and repeatedly grown in pure culture, the pathogen can induce the disease anew.

It is easy to see how these three postulates, suggested in the 19th century before knowledge of viral diseases, do not have to be fulfilled to reasonably establish a pathogen as the cause of a disease. The first postulate does not apply to a large number of pathogens that cause non-unique symptoms, such as respiratory infection or gastroenteritis. The second postulate is difficult to fulfill for several reasons, not least because a pathogen's ability to cause disease can vary widely

depending on the host's genetic makeup. The third postulate has been unreasonable for pathogens that can potentially cause severe disease and which do not have good animal models (e.g. mumps virus). Experimental bocavirus infections in consenting adults would probably not be dangerous but they would not necessarily represent the effects of primary infection in young children. However, the spirit of Koch's rules still holds in that scientists must demonstrate that a virus is not only associated with a disease but that it is actually the cause.

Several revisions to the original Koch's postulates have been proposed and among the most recent are the "molecular guidelines for establishing microbial disease causation" [107]. The guidelines are listed with Arabic numerals below and discussed to see how well the existing HBoV measures up with the criteria. Some of the lengthy original 7 guidelines have been split into smaller elements for the sake of clarity whereas others have been pooled together with minor text editing.

Rule 1) "A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease".

As mentioned, it is not reasonable to expect HBoV DNA in most cases of respiratory or gastrointestinal disease because a wide variety of human pathogens, both eukaryotic and viral, result in these symptoms. Most researchers have instead tried to investigate whether HBoV DNA is more often present in symptomatic than asymptomatic individuals but the results are often difficult to interpret because of differences in sampling techniques, the lack of truly asymptomatic controls and the prolonged shedding of HBoV1 (and possibly HBoV2-4) in the respiratory and fecal specimens.

We can nevertheless try to inspect epidemiological data from the studies in which the aforementioned confounding factors have been minimized. The focus of this inspection is on the presence or absence of viral nucleic acids in asymptomatic and symptomatic individuals. HBoV copy numbers are also mentioned to avoid repetition of data, but the importance of viral loads will be discussed in more detail later in the chapter.

To the present author's knowledge, only two studies have compared the occurrence of HBoV1 in children with and without respiratory disease and fulfilled five important criteria: i) the control cases were truly

asymptomatic and not e.g. undergoing elective surgery ii) the same sampling technique was used for both cohorts, iii) the samples were collected within the same time period, iv) the age distributions of the two groups were similar and v) the samples were collected from the same geographical area.

The first study by Linstow and colleagues was a prospective birth cohort study, which followed initially healthy infants for 1 year [65]. All children were from the same birth cohort ensuring identical age distribution, sampling technique, geography and time among symptomatic and asymptomatic children. Information on respiratory symptoms was collected from daily diaries to minimize the risk of recall bias. The presence of HBoV was determined with monthly nasal swabs. The authors found HBoV1 DNA in 8.2% of nasal swabs from symptomatic children with acute respiratory tract infection and in 8.6% of samples from asymptomatic children. The mean viral load in symptomatic individuals was 4-fold higher than in asymptomatic individuals without reaching statistical significance. However, the absence of statistical significance is difficult to assess without an estimate of statistical power. Symptoms in HBoV1 positive children without known coinfection with other respiratory viruses were generally mild, most commonly nasal discharge (90%) or cough (70%).

The second well-controlled investigation to meet all five aforementioned inclusion criteria was also a prospective cohort study [57], involving young children attending daycare. HBoV1 was reported in 44% of 45 asymptomatic children and 34% of 41 symptomatic children. The virus load in both groups was the same (within margin of error), 4.9×10^4 copies/ml among symptomatic and 4.6×10^4 copies among asymptomatic patients. HBoV infection alone was not associated with onset of illness and viral load did not correlate with severity of illness.

Unlike these two prospective studies, a study by Christensen and colleagues compared children with signs of RTI (median age 17 mo) to control children admitted for elective surgery (median age 31 mo). Even though the control children were not truly asymptomatic and their median age was almost twice the age of the RTI group, the study is highly relevant for this discussion. This is because Christensen and colleagues used HBoV1 mRNA rather than DNA as a marker of ongoing infection and both subject groups comprised entirely of subjects with HBoV1 DNA in NPA. The authors found that HBoV1 mRNA was detected in nasopharyngeal aspirates from 33 (25%) of 133 children with RTI

but in none of the 28 controls undergoing elective surgery. The authors also found that among all HBoV1 positive children, NPA DNA loads were significantly higher in children positive for HBoV1 mRNA in NPA than negative children (Figure 7). Both results were statistically very significant.
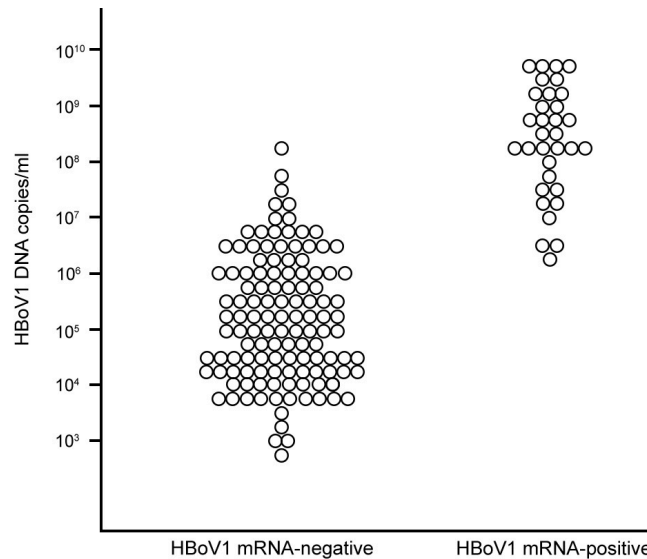
Figure 7. Distribution of HBoV1 DNA loads in nasopharyngeal aspirates either positive (n=33) or negative (n=128) for HBoV1 mRNA. Each dot indicates one sample. Digitally recreated from Figure 3 in *[98]* with adaptations.

Two studies assessing the roles of human bocaviruses in gastrointestinal disease also meet the five inclusion criteria. An Australian study [26] tested fecal samples from ~190 case-control pairs for HBoV1-3 DNA with qualitative PCR and found a significant association (17% vs. 8%) between HBoV2 detection and acute gastroenteritis (AGE). HBoV1 was not statistically associated with AGE (9% vs. 6%) and the number of HBoV3 positive samples was not high enough for meaningful conclusions. An English study analyzed ~2000 cases with gastroenteritis and an equal number of healthy controls [93] and found that HBoV2 was associated with gastroenteritis (1.4% vs. 0.8%) across children and adults. However, in children aged <5 years the prevalences in cases and controls were similar, 2.8% and 2.6%, respectively. HBoV3 occurred at ~1% frequency in the controls and patients aged <5 years and was not associated with gastroenteritis.

With slightly more relaxed inclusion criteria (different sampling intervals for cases and controls) the results of two other studies can also be inspected. Chen and colleagues [79] analyzed stools from ~400 cases and ~120

controls for HBoV1 DNA and found no difference in HBoV1 DNA positivity (3.5% vs. 3.5%). In the other study, Jin and colleagues [100] reported a statistically significant association between HBoV2 and gastroenteritis (20% vs. 12%). Both studies used quantitative PCR for virus detection but neither found statistically significant difference in DNA loads between cases and controls.

Statistically meaningful data are currently not available on the association of HBoV4 with human disease, since the virus was found so recently and occurs very rarely in respiratory or fecal specimens.

Rules 2 and 3) "Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased (i.e., with anatomic, histologic, chemical, or clinical evidence of pathology) and not in those organs that lack pathology. Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located".

Of the human specimen types studied so far (respiratory, urinary, fecal and tissue) HBoV1 DNA is undoubtedly most frequently found in respiratory specimens. Currently there is no histologic *in vivo* evidence for or against pathogenicity, as this would require lung or colorectal biopsies. However, the effects of HBoV1 infection have been studied *in vitro* using cultured pseudostratified human airway epithelium, the sole cell type currently known to support HBoV1 propagation. The first such study reported only slight morphological stretching of the cells [33]. There was no disruption of the cell layer, changes in cell density, ciliary movement or mucosal secretion compared to non-infected cells. However, the examination was done only 4 days after infection. A later and more thorough study continued observing the cells for up to 3 weeks post-infection and documented significant histological alterations starting approximately on day 7 post-infection [108]. These alterations included disruption of cell-to-cell tight junctions and loss of cilia. The tight junctions are likely to be the most important determinant of forming a virtually impermeable barrier to fluid, whereas cilia are important for the removal of mucus and exogenous particles from the respiratory airways. Compromise of these factors *in vivo* would contribute to the buildup of fluid and mucus in the lungs, the prominent features of pulmonary infiltrates and lung consolidation. There are

no published *in vitro* studies on the potential histological effects of HBoV2-4 infection.

Rule 4) "The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms".

The phylogenetically closest relatives of human bocaviruses that have been pathogenetically characterized are the canine minute virus and the bovine parvovirus. As we have seen in chapter 5.1.3, these viruses can cause respiratory or gastrointestinal disease, which is in line with the hypothesized roles of HBoV1 and HBoV2-4 in human disease. However, the implications of these roles should not be overestimated, since the human and animal bocaviruses are phylogenetically not very closely related (40% VP2 amino acid identity). We have already seen how remarkably the tissue tropism of HBoV1 and HBoV2-4 appear to differ despite sharing ~80% VP2 amino acid identity (chapters 5.2.4 and 5.2.5).

In contrast to the tentative supporting link in respiratory and gastrointestinal disease, the known biological characteristics of parvoviruses form a significant body of evidence *against* a causal link between HBoVs and cancer formation. The notion that HBoV1 infection could promote human cancer was recently put forward by Schildgen and colleagues [85] who found HBoV1 DNA in ~20% of colorectal and lung cancer tissues. The authors did acknowledge that actively replicating tumor cells could provide an optimal environment for HBoV1 since parvoviruses are believed to prefer dividing and proliferating cells. However, it should have also have been noted that parvoviruses are generally considered to suppress rather than promote tumor development. This oncosuppressive effect of parvoviruses has been demonstrated in laboratory animals (reviewed in [109]). Parvoviruses were found to protect the animals e.g. from chemically induced tumors, to suppress the development of established tumors and to make the animals less susceptible to tumor grafts. The tumor-suppressive effects are believed to result from the oncolytic effect of parvovirus infection [3].

Rules 5-7) "Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease and the copy

number of pathogen-associated nucleic acid sequences should decrease or become undetectable with resolution of disease. With clinical relapse, the opposite should occur. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship".

The limitations of simply comparing symptomatic and asymptomatic individuals for the incidence of HBoV1 DNA in respiratory specimens have already been discussed. However, information about viral copy numbers expands our ability to evaluate association between infection and disease also beyond well-paired cohorts of healthy and diseased individuals. For example, a Norwegian team of scientists [70] studied HBoV DNA levels in children with elective surgery (controls) and children admitted to hospital due to acute respiratory tract infection (cases). The authors found more HBoV1 positive respiratory samples in the controls than among the cases, but high ($>10^6$ copies/ml) NPA loads were strongly associated with infection of the lower respiratory tract. Very high levels of HBoV1 DNA in NPA ($>10^8$ copies/ml) also occurred exclusively among children with respiratory symptoms. On the other hand, the scientists who discovered HBoV1 examined children hospitalized for acute expiratory wheezing and found a positive association between high NPA DNA load and the absence of coinfecting respiratory viruses [56].

A Chinese [76] study examined children with X-ray-diagnosed lower respiratory tract infection and compared the severity of symptoms to the level of HBoV1 DNA. The authors reported a very clear difference in DNA loads between patients and healthy controls, as well as betweeevern mild and severe symptoms. Virtually all patients had viral loads in excess of 10^4 copies/ml cutoff level whereas all healthy controls had DNA levels below this level. The DNA load of the nasopharyngeal swab extracts of the healthy controls was on average 1000 fold lower than in the NPA samples from the patients.

HBoV1's pathogenetic role is also supported by three case reports that have found HBoV1 infection in children with life-threatening respiratory disease and adequately excluded other known viral, bacterial and fungal pathogens. HBoV1 DNA loads in these children were very high at the peak of symptoms (10^9 -10^{10} copies/ml) and decreased or became undetectable at the resolution of symptoms [110-112]. All three children had severe respiratory distress, all three showed lung infiltrates and two had abnormal collection of air

in the pleural space that separates the lung from the chest wall (pneumothorax). Chest radiograph of one of the three cases is illustrated in Figure 8.

Asymptomatic   HBoV1 pneumonia
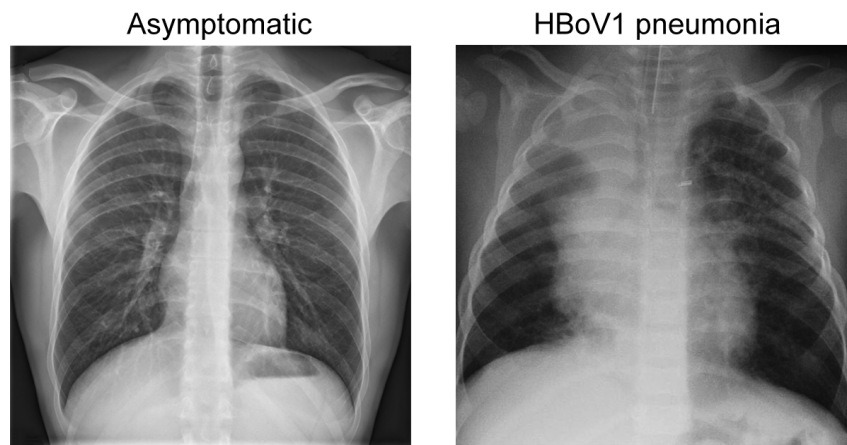
Figure 8. Chest radiographs of an asymptomatic individual and a 16-month-old Finnish boy with human bocavirus 1 pneumonia on 2nd day of hospitalization. Pulmonary infiltrations and atelectasis (collapse of lung tissue) of the right lobe can be seen. Copyright to the left side image has been licensed from 123RF Ltd (Hong Kong, China). Right side image reproduced from [110] with permission.

HBoV1 coinfection rate. It is reasonable to assume that HBoV1 can cause respiratory disease based on the data discussed so far. However, the overall clinical significance of the virus in comparison to other major respiratory pathogens in children hospitalized for respiratory symptoms is open for discussion. As we have seen, prospective or follow-up studies have not established a role for HBoV1 in respiratory disease among non-hospitalized children. On the other hand, HBoV1 infections in hospitalized children appear to have a remarkably high coinfection rate not only according to PCR analysis of respiratory specimens (the usefulness of which is undermined by DNA persistence) but also according to serodiagnosis and viremia. By comparing data from two studies on a group of children hospitalized for acute expiratory wheezing [56,113], one can see that only 25% of serologically diagnosed acute HBoV1 infections were monoinfections whereas the corresponding (PCR-based) figures for rhinovirus and RSV (diagnosed only by PCR) were at least twice as high, 77% and 50%. Equally high co-infection rate was noted by the Norwegian study on children admitted to hospital for respiratory tract infection. Of 18 children with HBoV1 viremia, 6 (33%) had no other respiratory virus in the NPA. It is therefore possible that infections by other respiratory viruses facilitate HBoV1 infection and therefore contribute to HBoV1's association with

respiratory disease. This would not be an unprecedented phenomenon, since influenza virus infection can mediate infection by vesicular stomatitis virus and Semliki Forest virus by enhancing the binding of these viruses to the respiratory epithelia [114]. RSV, on the other hand, is commonly detected with HBoV1 and can evidently suppress T-cell responses to itself and other antigens [115].

Summary. Existing data are in line with the notion that HBoV1 infections among immunocompetent individuals mostly result in mild symptoms (or are subclinical) but can sometimes cause serious symptoms requiring hospitalization. Well-controlled studies indicate an association between HBoV2 and gastroenteritis but causality has not been established. Not enough is currently known about HBoV3 or HBoV4 for a meaningful assessment of their role in human disease.

5.3   ORIGINAL ANTIGENIC SIN

5.3.1   INTRODUCTION

Based on research done on influenza, dengue and human immunodeficiency viruses (and possibly others), close structural similarity between related viruses may give rise to a immunologic phenomenon known as original antigenic sin (OAS). This phenomenon has potential to significantly modify immunological responses against later viral infections. As demonstrated in the results of this thesis, antibody responses against human bocaviruses also appear to conform to the OAS phenomenon. OAS does not only have the potential to influence the body's subsequent capability to stave off infections but also complicate their serological diagnosis. To paraphrase a recent editorial on OAS [116]: "what is original antigenic sin, what is its immunologic basis, and what sort of trouble is it getting us?"

Epidemiologist Thomas Francis, Jr (1900-1969) was a prolific vaccine researcher and the first to isolate influenza virus in the USA. While studying immune responses to influenza viruses, Francis and colleagues noted that sequential exposure to closely related influenza virus variants appeared to yield an antibody response that was significantly influenced by the first stimulus even if spaced several years apart. In one of the first and perhaps most

illustrative studies on the phenomenon [117], the effect of antibody absorption[iii] on hemagglutination inhibition (HAI) was studied with pre-vaccination serum pools from three different age cohorts; adults (30+ y), military recruits (17-25 y) and children (4-10 y). Each serum pool was separately absorbed with three different influenza strains; SW derived from the 1918 epidemic, PR8 isolated in 1934 and FM1 from the 1947 epidemic. The SW strain matched the type of strains circulating in the childhood of the adult cohort whereas PR8- and FM1-like strains had circulated in the childhood of the military recruits and children, respectively. The most interesting results were observed when absorption of the adult serum pool with the childhood SW strain also removed HAI activity against the PR8 and FM1 strains even though the adults had by necessity been exposed to these types of viruses after their early years. In contrast, HAI activity against the childhood SW strain was mostly retained in these individuals when the same serum pool was pre-absorbed with the later PR8 and FM1 strains. Similar results were observed from the vaccination of children with the SW and PR8 strains, since HAI activity towards both vaccines was entirely removed by absorbing the sera with the childhood FM1 strain. As with the adult subjects, activity toward the childhood FM1 strain was mostly retained after absorption with SW or PR8 strains.

 To confirm their results, the authors carried out consecutive infections of ferrets with different unattenuated influenza strains. Results from one such infection experiment are illustrated in Figure 9. The ferret was first infected with SW strain, 7 months later with a Weiss strain, and further 6 weeks later with a CAM strain. Serum was collected from the animal 2 weeks after each infection. When the final serum was absorbed with the strain of the first infection (SW), all HAI-active antibodies against all three strains were removed. In contrast, absorption with the second and third infecting strains only removed a relatively small proportion of the HAI activity of the first strain. Based on these and other similar results the authors concluded that exposure of the "virgin" ferret to the first infecting strain somehow prevented the formation of specific antibodies against the later two strains.

[iii] The process of removing or "tying up" undesired antibodies in an antiserum by allowing them to react with their antigens
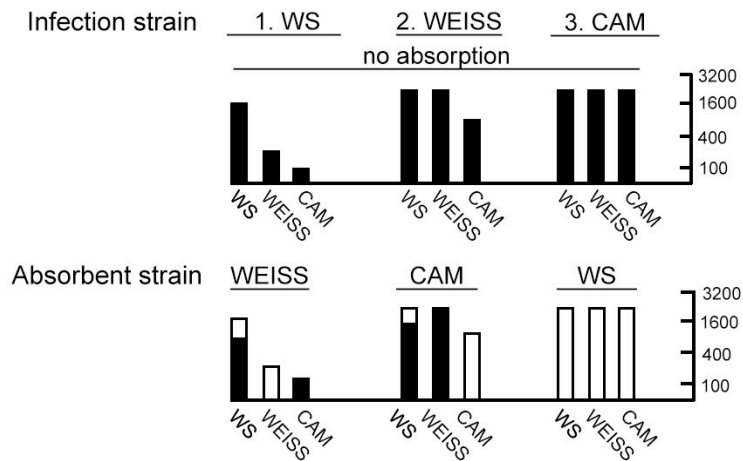
Figure 9. Hemagglutination-inhibition titers of ferret sera with and without antibody absorption after successive infections with 3 live strains of influenza H1N1 virus. In the upper image, the solid black bars represent the measured titer levels without antibody absorption. In the lower image, the black outlines show the same pre-absorption titers for visual reference, whereas the solid black bars represent the post-absorption titers. Digitally recreated from Figure 5 in study [117] (with adaptations).

Francis and colleagues also studied the effects of repeated homologous immunizations on children that grew up during the circulation of FM1-like influenza strains. The children were given 3 doses of monovalent vaccines containing Cuppet (isolated in 1950's), SW, PR8, or FM1 virus and the immune responses were measured without antibody absorption. As illustrated in Table 5, the secondary strains yielded highest antibody titers against the FM1 strain at the apparent expense of weaker responses to their own epitopes. Repeated exposures did not override the governing effect of the primary strain.

Table 5. HAI antibody titers in pools of sera obtained before and after administration of monovalent vaccines to children aged 4 to 10, born during the circulation of an FM1-like influenza strain. All titers were measured without antibody absorption. Adapted from [118].

Group	Vaccine	Bleeding	HAI titer with test strain			
			SW	PR8	FM1	Cuppet
1	SW	1	0	0	256	32
	SW	2	1024	128	>16384	1024
	SW	3	1024	64	>16384	1024
	-	4	2048	32	>16384	2048
2	PR8	1	0	0	1024	64
	PR8	2	64	2048	2048	128
	PR8	3	64	2048	2048	128
	-	4	64	1024	2048	128
3	FM1	1	0	0	256	32
	FM1	2	0	0	4096	512
	FM1	3	0	32	4096	512
	-	4	0	32	4096	512
4	Cuppet	1	0	0	256	32
	Cuppet	2	32	128	4096	1024
	Cuppet	3	0	64	8192	1024
	-	4	0	64	8192	512

In 1960 Francis wrote a commentary [119] in which he reflected the data collected during the last decade and termed the new phenomenon:

"The antibody of childhood is largely a response to the dominant antigen of the virus causing the first Type A influenza infection of the lifetime. As the group grows older and subsequent infections take place, antibodies to additional families of virus are acquired. But the striking feature is that the antibody, which is first established, continues to characterize that cohort of the population throughout its life. The antibody-forming mechanisms have been highly conditioned by the first stimulus so that later infections with strains of the same type successively enhance the original antibody to maintain it at the highest level at all times in that age group. The imprint established by the original virus infection governs the antibody response thereafter. This we have called the doctrine of original antigenic sin."

It is interesting that Francis' own description of OAS was rather vague and specifically highlighted the enhancement of antibodies against the first antigen rather than the lack of antibodies toward the second antigen.

Indeed, the concept of OAS appears to be rather elusive since some authors invoke it unnecessarily. For instance, a recent letter to the journal of Emerging

Infectious Diseases [120] discussed the recent H1N1 pandemic and drew attention to the fact that young individuals were at greater risk from acquiring H1N1 infection than older individuals. The authors suggested possible involvement of OAS in protecting the elderly [120]. The ensuing editorial commentary [116] asked whether OAS could be "an epidemiologic blessing in disguise". It is not clear why one would invoke OAS instead of simply immune memory to explain the age distribution of the H1N1 pandemic. On the other hand, one study reported mere heterologous boosting as evidence of OAS while, paradoxically, demonstrating robust activation of naïve B cells against the secondary antigen [121]. Needless to say, the heterologous boosting can be explained by stimulation of immune memory by shared epitopes between the primary and secondary antigen without any need to invoke a new phenomenon. Other studies have reported in support of OAS that, following the second immunization of primed animals, antibody or neutralizing titer against the secondary antigen was lower than against the primary antigen [122,123]. Direct comparison of titers after the second immunization overlooks the fact that secondary immunizations build upon existing immunological *memory* against the primary antigen, which can most likely be stimulated not only by identical epitopes but also by epitopes that are sufficiently similar between the two antigens. To clarify this issue, Figure 10 illustrates hypothetical antibody responses against consecutive immunizations with related antigens. As can be seen in the left side of the image, consecutive antibody responses can be expected to result in a lower overall antibody titer against the secondary antigen even in the absence of OAS. However, humoral response against the secondary agent would still yield an antibody response that is equal to that generated against the primary agent with respect to the titer of antigen-specific antibodies. Thus, a more reasonable comparison between the primary and secondary immune responses should be made by comparing the titer of specific antibodies against the primary antigen with the titer of specific antibodies against the secondary antigen.

For OAS to deserve its own term, it should be defined in a way that somehow distinguishes it from a typical immune memory response. In this thesis, the term is used to denote the inhibited activation of naïve B or T cells[iv]

[iv] A naive B or T cell refers to a cell that has not been exposed to an antigen. Upon antigen exposure, it proliferates into many clones. Some of the clones are transformed into effector cells that will defend the body in an immune response whereas others are transformed into long-lasting memory cells.

toward a secondary antigen due to pre-existing immunity toward a previously encountered primary antigen.
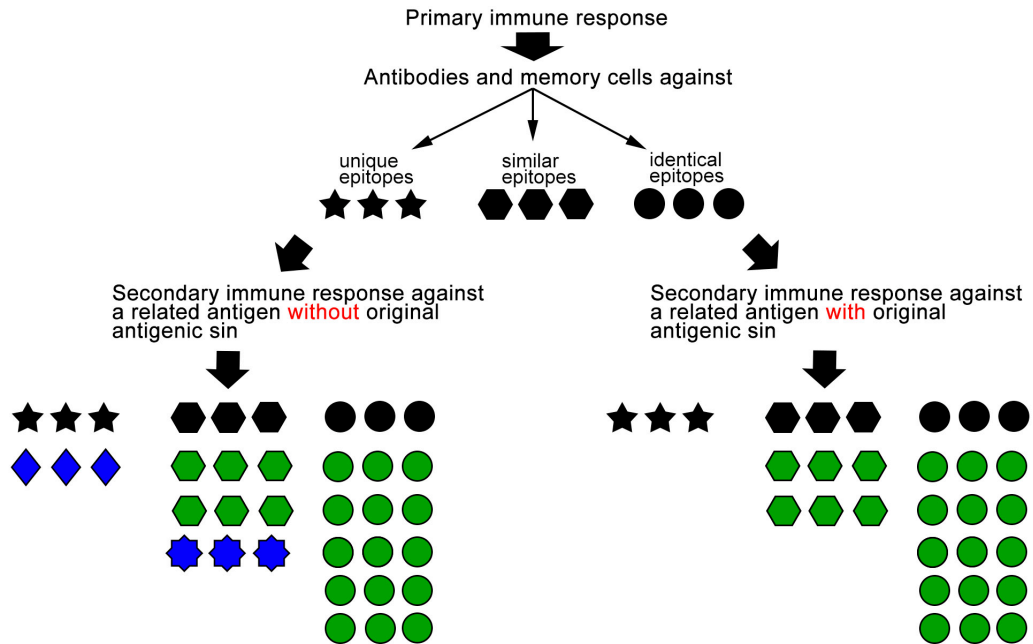
Figure 10. Hypothesized antibody responses to consecutive immunizations with two related antigens in the absence and presence of original antigenic sin. Black and blue symbols represent antibodies produced by the activation of <u>naïve</u> B cells against the primary and secondary antigens, respectively. Green symbols represent antibodies produced by the activation of <u>memory</u> B cells (from the 1st infection) by the secondary antigen. The large number of green circles relative to the number of hexagonal symbols symbolizes the ability of the fully matching epitopes to stimulate memory B cell receptors with higher efficiency than partially matching epitopes. Note: OAS is demonstrated here as complete inhibition of naïve B cell activation but the real phenomenon probably gives rise to a spectrum of inhibition depending on immunological circumstances.

5.3.2 POTENTIAL MECHANISMS OF INDUCTION AND EVASION

One possible mechanism for the formation of OAS is the inhibition of naïve B cell recruitment through the action of Fcγ receptors. Fcγ receptor is a protein found on the surface of certain cell types the immune system, such as B and T lymphocytes, macrophages and neutrophils. Its name is derived from its binding for the constant region (Fc) of IgG molecules. There are different types of Fc receptors, each binding to different class of antibodies. The Greek letter γ indicates that Fcγ receptor specifically binds to IgG. As illustrated in Figure 11, Fcγ receptors may inhibit naïve B cell activation because the co-engagement of the Fcγ receptors and the B cell receptors (BCR) by antigen-IgG complexes

blocks the activation signal from the BCR [124-126]. Although not shown experimentally, at least four other mechanisms could also take part in the suppression of primary immune responses: (1) masking of new epitopes by the binding of antibodies to closely located conserved epitopes, (2) competition for antigen by higher affinity memory cells, (3) annihilation of antigen by phagocytotic cells or the complement system before it can reach the naïve B-cells in sufficient quantity and/or (4) limitation of the propagation of the secondary pathogen so that less antigen is available for the stimulation of the immune system. This last potential mechanism, the limited availability of antigen, may be brought by B or T cells targeting conserved (and in the case of B cells, neutralizing) epitopes in the secondary antigen. Thus, based on points 2 and 4, OAS may be partially mediated by cross-immunity.
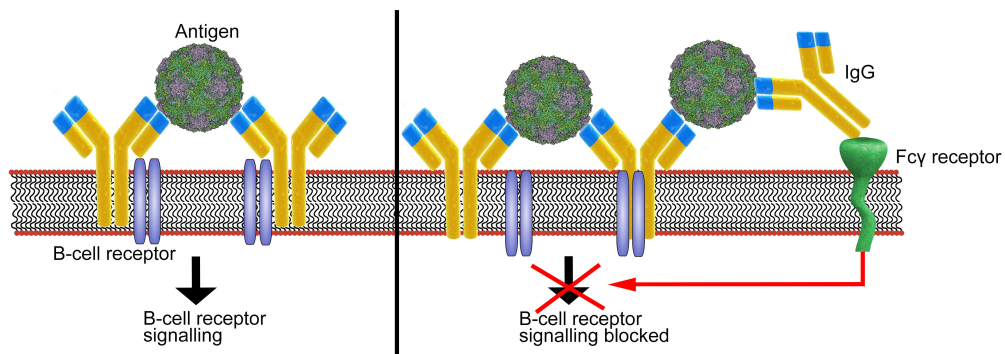
Figure 11. Inhibition of naïve B-cell activation by cross-linking of B-cell receptors and Fcγ receptors. Copyrights to reproduced image elements (viral capsid, antibody molecule and the receptor molecule) have been licensed from 123RF Ltd (Hong Kong, China).

All these possible mechanisms of OAS could potentially be evaded by increasing the load of the secondary antigen sufficiently to "flood" the inhibitory mechanisms. This could have a significant effect on the results obtained from immunization experiments and cause discrepant results with native infections, in which the antigen loads are several orders of magnitude smaller. Large antigen doses could play some role in a recent study [127] that called into question the significance or perhaps even existence of OAS. The authors used vaccinated human volunteers to isolate human immunoglobulin variable regions from individual antibody-secreting plasma cells to study the affinity of monoclonal antibodies against different strains of influenza. Even though all volunteers had been vaccinated against other influenza strains prior to 2007, most of the mAbs showed highest affinity toward the 2007 vaccine strain,

indicating efficient naïve B cell activation. Based on these results, the authors suggested that OAS does not seem to be a common occurrence in healthy adults receiving influenza vaccinations.

In addition to sufficiently low antigen loads, it is also possible that manifestation of OAS on the B and T cell level somehow requires live virus infections rather than immunizations with an inactivated virus. This was suggested by Kim and colleagues [128], who studied the effects of OAS in mice by vaccinating the animals with whole inactivated influenza viruses or sequentially infected with live mouse-adapted viruses. They found no firm evidence of OAS in the sequentially vaccinated mice in terms of serum neutralization titer or HAI activity, but profound OAS induction in the sequentially infected mice was detected. Secondary infection of PR8-primed mice with the FM1 strain of influenza A resulted in neutralization and HAI titers against FM1 ten-fold lower than in primary infection of non-primed mice.

5.3.3   CLINICAL SIGNIFICANCE

The clinical implications of OAS are easy to understand, since inhibition of activation of naïve lymphocytes may allow the secondary virus to escape neutralizing immune responses. A relatively recent study illustrated the practical effects of OAS. In this study mice were sequentially immunized with sublethal doses of live influenza PR8 and FM1 strains (in this order) and then giving them a lethal dose of FM1 [128]. To compare the results to a "regular immunization", they used mice that first received PBS, then a sublethal dose of live FM1, and finally a lethal dose of FM1. Non-vaccinated controls first received two sequential doses of PBS and then a lethal dose of FM1. The results are illustrated in Figure 12.
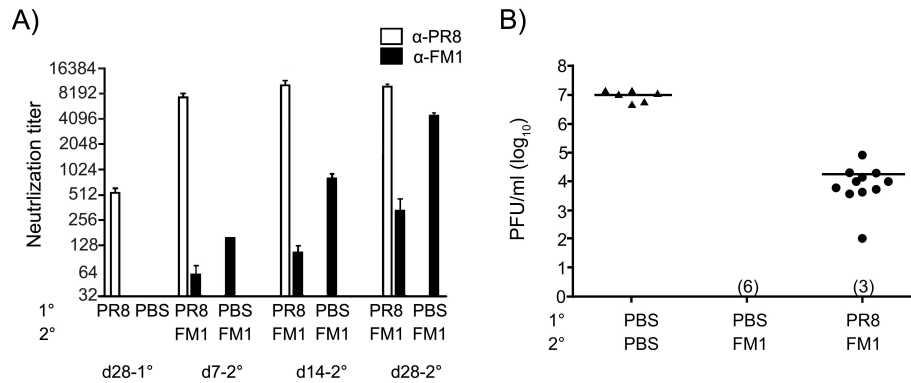
Figure 12. Induction of original sin in mice by sequential infection with $0.1 \times LD_{50}$ of PR8 and FM1 viruses (LD_{50} is the dose required to kill half the members of a test population). The mice were first infected with PR8 (or treated similarly with phosphate buffered saline [PBS]) and the first follow-up sample was taken 28 days later (d28; image A). The mice were then infected with FM1. Follow-up samples were collected on days 7, 14 and 28 and analyzed for neutralization titers (image A; error bars represent the standard error of the mean). After the last follow-up sample, the mice were challenged again with FM1 but this time with a lethal dose (FM1; $100 \times LD_{50}$). A few days after the lethal dose, the lungs of the mice were harvested and assessed for FM1 titers (image B). Bracketed numbers indicate the number of mice with no detectable FM1 in lungs. Each data point represents an individual animal. Error bars represent standard error of the mean. Adapted from [128] with permission (copyright 2009, The American Association of Immunologists, Inc).

As shown in the Figure, the naïve, unimmunized control mice that received only PBS exhibited lung titers of 10^7 plaque-forming units (pfu) per ml upon the final FM1 challenge, whereas the mice that were first immunized with sublethal dose of FM1 cleared the virus completely. In contrast, the mice that had sequentially received a sublethal dose of PR8 and FM1 developed lung titers of 10^4 pfu/ml. The results demonstrate that FM1 immunization among PR8-primed mice left the animals vulnerable to later FM1 infection, whereas FM1 immunization of "virgin" mice resulted in complete protection. However, one should note that the primed mice were partially protected against the infection and not worse off than the virgin animals with respect to the ensuing viral titers.

Antibody-dependent enhancement of infection (ADE) may be one mechanism of the clinical effects of OAS. According to ADE, non-neutralizing IgG molecules bound to virus particles promote infection of Fc-bearing cells such as monocytes and macrophages through endocytosis (Figure 13). OAS may mediate ADE by inhibiting the formation of neutralizing antibodies against the second infecting virus strain, especially if the neutralizing antibodies are mostly directed toward non-conserved epitopes that require the activation of naïve B cells. This would give rise to a pool of antibodies which mainly serve to enhance

viral access into Fc-bearing cells without obstructing the spread of the virus. ADE was first suggested by R. A. Hawkes who showed that virus-specific antisera were able to increase the infectivity of certain arboviruses in cell cultures [129]. A later study with a dengue virus showed that the infection-enhancing factor was an IgG-class antibody. I*n-vitro* ADE by sub-neutralizing IgG has also been demonstrated for Aleutian mink disease parvovirus [130] and several influenza A subtypes [131].
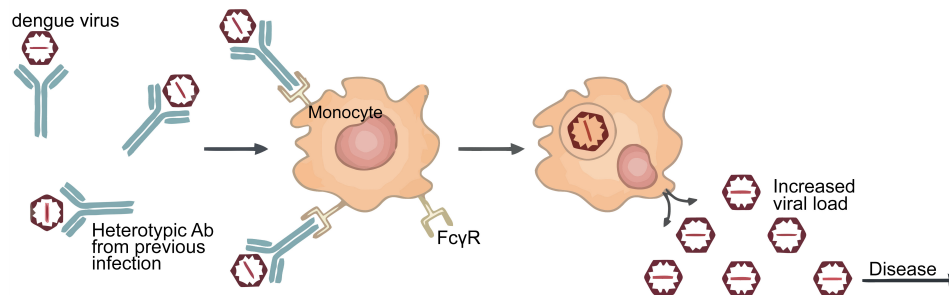
Figure 13. Model for antibody-dependent enhancement (ADE) of dengue virus replication [132]. Reprinted in adapted format by permission from Macmillan Publishers Ltd: Nature, copyright 2007.

ADE has been hypothesized to be partially responsible for the increased risk of life-threatening dengue haemorrhagic fever/dengue shock syndrome in secondary dengue virus infections [133]. This potentially fatal disease is characterized by increased vascular permeability leading to hemoconcentration and hypovolemic shock. The clinical significance of ADE has been demonstrated in a mouse model [134]. Passive administration of anti-dengue antibodies to the mice enhanced dengue virus infection and gave rise to several hallmarks of severe dengue disease, including vascular leakage, reduction of blood platelets and increased viral loads in serum and tissue phagocytes. In contrast, genetically engineered monoclonal antibodies that could no longer bind FcγR had preventive and therapeutic efficacy against a dengue virus challenge.

6 AIMS OF THE STUDY

When this thesis project was initiated, HBoV1 was the only human bocavirus known and the diagnosis of HBoV1 infections relied on PCR analysis of respiratory or serum specimens. Moreover, nothing was known about HBoV immunity. The initial aim of the study was therefore

  1) To study HBoV1-specific B-cell responses and assess their diagnostic use in young children with respiratory disease.

The subsequent discovery of HBoV2-4 introduced three new human bocaviruses that were phylogenetically closely related to HBoV1 and to each other. The immunological properties of HBoV2-4 and the potential cross-reactivity of anti-HBoV antibodies in serological tests were unknown. Furthermore, the detection of these viruses relied on nested PCR assays, which are laborious and inappropriate for accurate quantitation of viral DNA. The other aims of the study were therefore

  2) To develop and evaluate real-time quantitative PCR assays for the detection of HBoV1-4 DNA

  3) To develop antibody assays for the study of HBoV1-4 immune responses

  4) To assess the cross-reactivity of HBoV1-4 in serological assays

  5) To assess the extent of HBoV1-4 circulation in Finnish children and adults

  6) To determine the longevities of IgG and IgM responses against the four HBoVs

  7) To study the immunological interactions of consecutive HBoV1-4 infections

7   MATERIALS AND METHODS

7.1   SUBJECTS

7.1.1   ADULTS AND CHILDREN WITH DIARRHEA (I)

Stool samples were collected from 250 Finnish children or adults with symptoms of diarrhea [135]. Of the 250 individuals, 100 had travelled outside Northern Europe within 1 month prior to sample acquisition. The median ages of the travellers and non-travellers were 31 years (range <1 to 74 years) and 53 years (range <1 to 95 years), respectively. The travel destinations were Europe (54%), Asia (29%), Africa (9%), South America (2%) or unknown (2%). Of all subjects, 33 were children aged 0 to 10 years, of whom 12 (36%) had travelled abroad. The samples were submitted to a diagnostic laboratory for routine testing and provided to us without identifying information.

7.1.2   CHILDREN WITH ACUTE WHEEZING (II, III)

The study cohort consisted of children with acute expiratory wheezing [136]. At hospital admission, a nasopharyngeal aspirate (NPA) was collected [136]. Blood samples were collected at the time of admission and 2–3 weeks after discharge.

The NPA samples of the entire cohort of 259 children have been analyzed in an earlier virus etiology study [56] for the following 16 viruses: HBoV1; adenovirus; coronaviruses 229E, OC43, NL63 and HKU1; enteroviruses, rhinoviruses, influenza viruses A and B, human metapneumovirus, parainfluenza viruses 1-4 and respiratory syncytial virus. Of these 259 children, 49 were HBoV1 PCR positive in NPA.

Paired serum samples from 252 of the 259 children were available for this thesis project. For Study II, we included all the 49 children with HBoV1 DNA in NPA and, as controls, 68 randomly selected children without HBoV1 DNA in NPA. For Study III we included all 252 children. The demography of both subpopulations and the entire cohort of 252 children are illustrated in Table 6. The Ethics Committees of Turku University Hospital (Turku, Finland)

and Helsinki University Hospital (Helsinki, Finland) approved the study protocol.

Table 6. Demography of the children with acute wheezing.

	No of patients	Median age	Age range
All children; Study III	252	1.6 y	2 mo - 15 y
HBoV1 NPA PCR positive subset; Study II	49	2.2 y	5 mo − 11 y
HBoV1 NPA PCR negative subset; Study II	68	2.5 y	5 mo − 12 y

7.1.3 FINNISH MEDICAL STUDENTS AND PAKISTANI BLOOD DONORS (III)

Serum samples were obtained from 115 healthy Finnish medical students (median age 23 years; range 21–32 years) [113,137] and 80 Pakistani blood donors (median age 20 years; range 18– 20 years). Written informed consent was obtained from the study participants.

7.1.4 "DIPP" CHILDREN WITH GENETIC SUSCEPTIBILITY TO TYPE 1 DIABETES (IV)

The study population consisted 109 constitutionally healthy children from whom serum samples (n=1961) were obtained with 3- to 6-month intervals from infancy to up to 13 years of age. The children were participants in a population-based Diabetes Prediction and Prevention (DIPP) study and were genetically susceptible to type 1 diabetes [138]. These children have been previously examined from infancy up to adolescence by HBoV1 immunoglobulin (Ig)M, IgG, and IgG-avidity enzyme immunoassays and quantitative PCR (qPCR) [139].

Of the entire set of 1961 serum samples, 1943 were available for this thesis project. The available samples were reanalyzed for HBoV1 antibodies and analyzed for the first time for HBoV2-4 antibodies and DNA. The patient demographic and sampling parameters are summarized in Table 7. The ethics

committee of the Hospital District of Southwest Finland approved the study protocol.

Table 7. DIPP cohort demography and sampling.

	Median	Mean	Range
No of samples	17	18	17-27
Age at the beginning of sampling	0.3 y	0.3 y	0.2 y – 0.9 y
Age at end of sampling	8.5 y	8.0 y	4.0 – 13.2 y
Sampling interval when age < 2 y	96 d	110 d	55 - 484 d
Sampling interval when age ≥ 2 y	182 d	197 d	92 - 849 d

7.2   HBoV1-4 MULTIPLEX qPCR ASSAY (I)

Assay principle. There are two main types of qPCR assays: those based on DNA binding agents and those based on Förster (or fluorescence) resonance energy transfer (FRET). The former can be problematic because the dyes bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, potentially resulting in false positive results and overestimation of the target concentration. FRET-based methodology offers better assay specificity since a positive result requires the specific binding of at least three oligonucleotides. The oligonucleotides furthermore have to bind the *same* target molecule with sufficient affinity, be in correct orientation relative to one another and spaced sufficiently close for a positive signal to be generated. Different variations of the FRET-based methodology exist but the assay in this thesis was based on the "hydrolysis probe" approach that is illustrated in Figure 14.
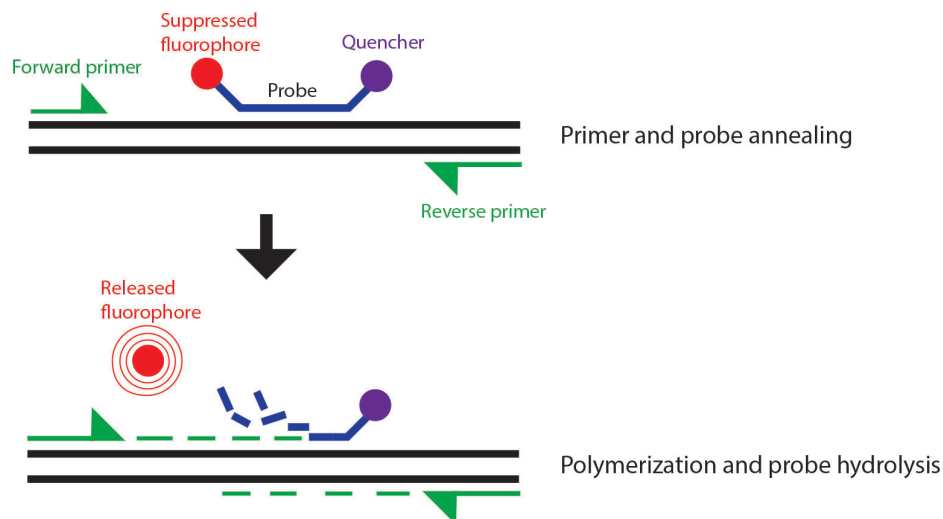
Figure 14. Principle of hydrolysis probe based DNA quantitation. The quenching moiety prevents emission of fluorescent light by the fluorophore in an intact probe molecule by converting incoming radiomagnetic radiation into heat or another wavelength that is not registered by the detector. However, the fluorophore is released from the quencher's influence when the polymerase reaches the annealed probe and degrades it by catalyzing phosphodiester bond hydrolysis. Each round of amplification increases the amount of probe template and therefore the amount of fluorescence through the build-up of the non-suppressed reporter molecules.

Plasmid controls. Development of qPCR assays for HBoV1-4 DNA detection required plasmid constructs containing at least partial virus genomes for use as amplification templates. For this purpose we selected parts of the left-hand untranslated region and the NS1 gene of the human bocavirus genomes, corresponding in HBoV1 to nucleotides (nt) 98 to 388 (GenBank accession number EU984245). The HBoV2 and HBoV3 sequences corresponding to the GeneBank entries FJ170279 and EU918736, respectively, were synthesized by GenScript corporation (NJ, USA). The HBoV4 sequence corresponding to the GenBank entry FJ973561 was synthesized by PCR with two long overlapping primers as template (Figure 15 & Table 8) and cloned into pJet1.2 (Fermentas, Burlington, Canada) by standard cloning practices. A near-full-length HBoV1 clone (pST2; GenBank accession no DQ000496) was a kind gift from Tobias Allander (Karolinska Institute, Sweden). PCR specificity was evaluated with cloned full-length of near-full-length genomes of parvovirus B19 genotypes 1-3 (GenBank accession numbers AY504945.1, AY044266 & NC_004295.1), Torque teno virus (AY666122.2), BK virus (DQ305492) and JC virus (AB372036).
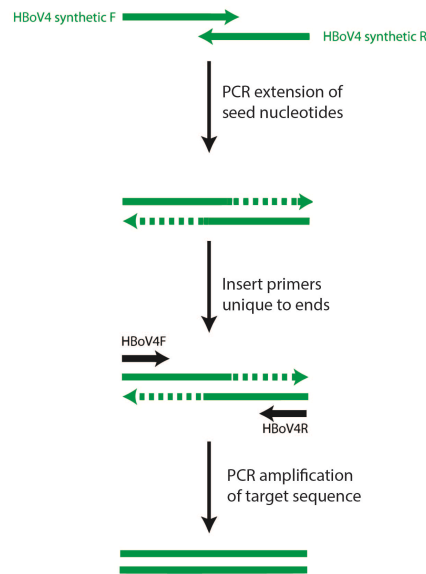
Figure 15. Synthesis of the HBoV4 insert by assembly PCR.

Primers and hydrolysis probe. The intra- and interspecies variation of human bocavirus genomes was studied by aligning nine HBoV1 sequences, nine HBoV2 sequences, four HBoV3 sequences, and one HBoV4 sequence. The HBoV1 and -2 strains were selected from a wide variety of geographical locations (10 countries). For HBoV3 and -4, we used all near-full-length genomic sequences that were available in 2010. The GenBank accession numbers and geographical origins of these sequences have been listed in Supplementary Table 3.

The sequence analysis revealed a genome region that was fully conserved among all known bocavirus species and strains (Figure 16). A DNA probe conjugated with minor groove binder (MGB), 5' FAM fluorophore and 3' black hole quencher 1 (BHQ1) targeted this region. The flanking (non-conserved) DNA regions in turn were targeted with five primers.

HBoV1 CCTATAAGCTGCTGCACTTCCTGATTCAATCAGACTGCATCCGGTCTCCGGCGAGTGAACAT
HBoV1F
H1234probe
HBoV2 CCTATAARCCRATGCACTTCCGCATYTCGTCAGACTGCATCCGGTCTCCGGCGAGTGAACAT
HBoV3 CCTATAAACCGATGCACTTCCGCATTTCGTCAGACTGCATCCGGTCTCCGGCGAGTGAACAT
HBoV4 CCTATAAACCGATGCACTTCCGCATCTCGTCAGACTGCATCCGGTCTCCGGCGAGTGAACAT
HBoV234F

CTCTGGAAAAGCTCCACGCTTGTGGTGAGTCT-ACTATGGCTTTCAATCCTC
HBoV1R
CTCTGGGAAGAGCTCCACGCRCGTGGTGAGTGACACTATGGCCTTTTCTGCTC
HBoV24R
CTCTGGGAAGAGCTCCACRCTTGTGGTGAGTCAAATTATGGCTTTCAATCCAC
HBoV3R
CTCTGGAAGAGCTCCACGCTTGTGGTGAGTGACACTATGGCCTTTTCTGCTC

Figure 16. DNA sequence alignment of the HBoV1 to -4 genome segments used for the qPCR. The alignment has been split into two parts as indicated by the arrow. Intraspecies sequence variation is underlined and shown as degenerate bases according to the symbols of the International Union of Pure and Applied Chemistry (IUPAC; R = A+G; Y = C+T). Fully conserved nucleotides are shown without underlining, whereas nucleotide differences between the species are in bold. Annotated (blue and brown) arrow symbols show the positions and directions of the primers and the probe. For HBoV2 and HBoV4, direct PCR product identification is possible by PCR product sequencing to distinguish the three-nucleotide difference.

In multiplex format the conserved probe together with the five primers were designed to detect all published types of human bocaviruses. In a singleplex format, the same primers could be used in pairs to distinguish between HBoV1, HBoV3 and HBoV2/4. The primer and probe sequences are listed in Table 8. The primer names indicate their usage. For instance, primer HBoV234F is the forward (sense) primer shared by HBoV2, -3 and -4 qPCRs while the HBoV1R reverse (antisense) primer is only applicable to HBoV1 PCR.

Table 8. Primers used for the development of HBoV1-4 qPCR multiplex assay.

Name	Sequence (5'->3')
HBoV1F	CCTATATAAGCTGCTGCACTTCCTG
HBoV1R	AAGCCATAGTAGACTCACCACAAG
HBoV234F	GCACTTCCGCATYTCGTCAG
HBoV3R	GTGGATTGAAAGCCATAATTTGA
HBoV24R	AGCAGAAAAGGCCATAGTGTCA
Probe	FAM-CCAGAGATGTTCACTCGCCG-MGB
HBoV4, synthetic F	GCACTTCCGCATCTCGTCAGACTGCATCCGGTCT CCGGCGAGTGAACATCTCTGGAAAGAGCT
HBoV4, synthetic R	GAGCAGAAAAGGCCATAGTGTCACTCACCACAA GCGTGGAGCTCTTTCCAGAGATGTTCACT
HBoV4F	GCACTTCCGCATCTCGT
HBoV4R	CACTATGGCCTTTTCTGCTC

The HBoV2 and HBoV4 primer binding sites in the HBoV2 and HBoV4 plasmids are identical except for a single-nucleotide difference in the forward primer (Figure 16). Moreover, in silico secondary-structure predictions with Mfold [140] showed no major difference between the amplified regions of the two sequences (data not shown). This minor difference was taken into account by using a degenerate nucleotide. Preliminary experiments indicated that the two plasmids were indistinguishable with respect to assay sensitivity and reproducibility. Consequently, the results reported in the results of this thesis (chapter 8.1) for HBoV2 and HBoV4 are designated "HBoV2/4" and were acquired with the HBoV2 plasmid.

Real-time PCR and sequencing. All quantitative PCRs were done using Stratagene Mx3005p qPCR device (Stratagene, CA). The reactions consisted of 1x TaqMan Universal Master Mix (Applied Biosystems, CA) with AmpErase uracil-N-glycosylase[v] (UNG), 0.6 μM concentrations of primers, 0.3 μM FAM-labeled probe and 2 μl of template in 25 μl total volume. Only one primer pair was used in singleplex reactions whereas the multiplex reaction included all five primers.

UNG was allowed to degrade potential carryover PCR products for 2 min at 50°C. After further 10 min at 94°C, 40 cycles of amplification (95°C for

---

[v] Uracil N-glycosylase (UNG) is an enzyme used for the elimination of carryover PCR products. The dUTP nucleotide is substituted for the dTTP nucleotide when using this enzyme, resulting in dUTP containing PCR amplicons. A short pre-PCR incubation step in subsequent PCR amplifications will allow the UNG to digest dUTP-containing DNA.

15 s, 60°C for 1 min) were performed. All runs included plasmid and no-template controls. To generate baseline-corrected fluorescence data, baseline fluorescence was determined by the Mx4000 software version 3.01 (Stratagene) baseline algorithm. The cutoff for quantification cycle determination was calculated as 20 times the standard deviation of the baseline fluorescence in cycles 5 through 9. The fluorescent reporter signal from the FAM channel was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations between samples. Rigorous laboratory procedures were followed to prevent PCR contamination, including separate spaces for handling of samples, master mix components, and plasmid templates. Aerosol resistant filter tips and disposable racks were used. All runs included water controls, which remained negative throughout the study.

The PCR products were enzymatically purified for sequencing using ExoSAP-IT (USB Corporation, Ohio, USA). Sequencing was done at the sequencing core facility of Haartman Institute. The sequences were compared to GenBank database entries with Geneious 4.5 (Biomatters Ltd, Auckland, New Zealand).

7.3   HBoV VP2 EXPRESSION

7.3.1   PROKARYOTIC HBoV1 VP2 EXPRESSION (II)

Cloning. The entire HBoV1 VP2 and the unique region of HBoV1 VP1 (VP1u) genes were amplified by PCR from a plasmid containing a near full-length HBoV clone (GenBank accession no. DQ000496). By utilizing restriction sites incorporated in the PCR primers (Table 9), the amplicons were cloned into the fusion expression vector pET23b (Novagen; Figure 17). The vector incorporates an N-terminal T7 tag and a C-terminal hexa-histidine tag (6xHis-tag) into the recombinant protein to facilitate detection and purification.

Table 9. Primer sequences used to amplify the capsid genes for cloning.

Amplicon	Primers
VP1u	5'-ATG**GAATTC**GATGCCTCCAATTAAG -3'
	5'-GTC**CTCGAG**TTTTGAGGTTCCTGGTTTAG-3'
VP2	5'-GAAC**GAGCTC**CATGTCTGACACTGAC-3'
	5- GAGGCTTATA**CTCGAG**CAACACTT- TATTG -3

Note: VP1u, unique part of virus protein 1; VP2, virus protein 2. The restriction sites GAATTC, CTCGAG and GAGCTC (marked with bold) are recognized by EcoRI, Xho I and SacI, respectively.
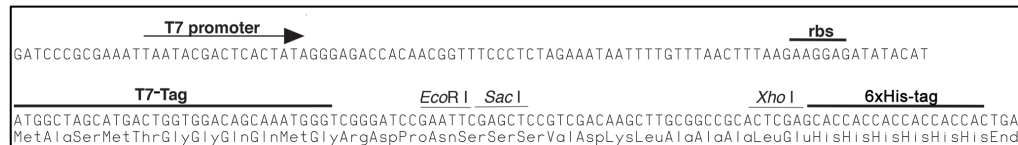

Figure 17. The cloning/expression region of the pET-23b vector. Rbs, ribosome binding site.

Expression host. The expression vector construct was chemically transformed into the *Escherichia coli* (*E.coli*) expression host BL21(DE3)pLysS (Invitrogen). The (DE3) designation indicates that the strain carries a chromosomal copy of the T7 RNA polymerase gene, which is inducible by IPTG. The pLysS designation in turn indicates that the plasmid produces T7 lysozyme, a natural inhibitor of T7 RNA polymerase. The presence of T7 RNA polymerase and its inhibitor together provide tight control of recombinant protein expression, thus inhibiting elimination of the insert from the cell population by natural selection.

Protein preparation. For protein expression, 0.5 l of Terrific broth or Luria broth were inoculated with recombinant *E.coli* and grown at 37°C until the culture reached an OD_{600} of 0.5. The cells were then induced for 3 h with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and harvested by centrifugation. The pellet was resuspended in 25 ml of phosphate buffered saline (PBS) and lysed by sonication on ice. The His_6/T7-tagged proteins were purified under denaturing conditions by nickel-nitrilotriacetic acid (Ni-NTA; Qiagen, CA, USA) affinity chromatography in accordance with the instructions of the resin manufacturer.

7.3.2 EUKARYOTIC HBoV1-4 VP2 EXPRESSION (III)

Gene synthesis. Genes coding for the HBoV2-4 VP2 were synthesized in the pUC57 cloning vector by GenScript Inc. (NJ, USA) based on the GenBank amino acid sequences ABW79868, ACR15792 and YP_002916063, respectively. To maximize expression of the VP2 genes in *Spodoptera frugiperda* (SF9 strain) or *Trichoplusia ni* (High Five strain) insect cells, the genes underwent sequence optimization by GenScript's proprietary software to improve codon usage and reduce disadvantageous mRNA secondary structures. All sequence modifications were such that the amino acid sequences of the original genes were not affected. The initiation codons were also placed in an optimal translation initiator context [141] and a non-translated sequence shown to promote recombinant protein expression was inserted immediately upstream of the translation initiation codon [142]. The insert design is illustrated in Figure 18 with the HBoV3 construct. The nucleotide sequences of the sequence-optimized HBoV2-4 VP2 gene sequences have been deposited to GenBank with the respective accession numbers KF420120, KF420121 and KF425523. The cloning of the HBoV1 VP2 baculovirus vector construct has been described previously [113].

The bovine parvovirus VP2 gene was amplified by PCR from bovine serum with the sense primer 5'-TCAA**AGATCT**ATGGAGGTATCAAATGATATACC-3' and antisense primer 5'-TTGC**AGATCT**TCACAGGACTTTGTGGTGATTGA-3'. The primers contain the Bgl II restriction site for cloning (marked with bold). No additional elements were added to the bovine parvovirus VP2 sequence to increase its translation in insect cells.
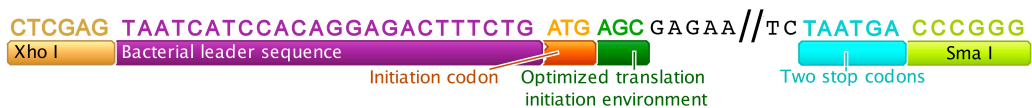
Figure 18. Illustration of the cloning strategy of HBoV VP2 genes with the HBoV3 sequence as an example. The codon-optimized VP2 gene was preceded by a bacterial leader sequence shown to promote expression of recombinant proteins in insect cells [142] and the translation initiation codon environment was optimized for efficient recombinant protein expression [141]. Two slash characters symbolize the unshown portion of the gene. A double stop codon was added to prevent translational read through.

Cloning. The optimized genes were cloned into the baculovirus transfer vector pAcSG2 (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) under the control of the strong polyhedron promoter by standard cloning practices. To produce a recombinant baculovirus, SF9 insect cells were co-transfected with the transfer vector DNA and linearized baculovirus DNA (BaculoGold, Pharmingen, San Diego, CA, USA) using the Fugene6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) as recommended by the manufacturer. The generation of recombinant baculoviruses is illustrated in Figure 19.
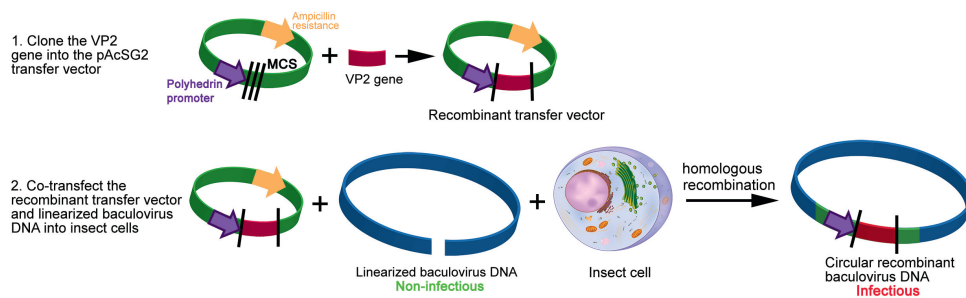
Figure 19. Generation of recombinant baculoviruses. MCS, multiple cloning site. Copyright to the insect cell image has been licensed from 123RF Ltd (Hong Kong, China).

Protein expression, purification and biotinylation. For production of recombinant proteins, the insect cells were infected with recombinant baculoviruses and incubated for 3 days at 27°C. The cells were pelleted by centrifugation at 2500×g for 10 minutes at +4°C and resuspended in PBS with a protease inhibitor mixture (Complete Mini; Roche Diagnostics, Basel, Switzerland) and lysed by sonication. VLPs were purified from the lysate supernatant by ultracentrifugation in a 28% cesium chloride (CsCl) gradient (at 100,000 x g for 24 h, +4°C).

7.4 IMMUNOASSAYS

7.4.1 WESTERN BLOT

Recombinant HBoV1 VP2 and VP1u protein samples purified by Ni-NTA affinity chromatography were separated by SDS-PAGE and transferred onto

nitrocellulose membranes (Protran BA 85; Whatman, Schleicher & Schuell). The membranes were blocked for 30 min with blocking buffer (5% dry milk and 0.2% Triton-X-100 in PBS) at room temperature. The blocked membranes were incubated for 1 h at room temperature with human serum samples diluted in the blocking buffer (IgG, dilution 1:50; IgM, dilution 1:30). After each step, the sheet was washed 3 times for 10 min each with PBS containing 0.05% polysorbate 20 (PBSP). Antibody-conjugate complex was visualized by horseradish peroxidase conjugated antihuman IgG or IgM (Dako, Glostrup, Denmark; 1:500 dilution) with 3,3'-diaminobenzidine as the substrate and H_2O_2 as the oxidizing agent. Stained bands were scored negative or positive before knowledge of PCR results. Equivocal results were considered negative.

7.4.2 ENZYME IMMUNOASSAYS

IgG EIA without antibody absorption. The IgG assays were done in indirect format as illustrated in Figure 22. The biotin-streptavidin[vi] system was used to avoid direct contact between the antigen and the polystyrene surface. This has the potential to significantly alter the three-dimensional structure of conformational antigens [143]. Covalent binding of biotin to the antigens was done using the EZ-Link Sulfo-NHS-LC-biotinylation kit (Pierce, Rockford, USA) as recommended by the manufacturer. The streptavidin coated plates were provided by Thermo-Scientific (Rockford, IL).

[vi] Biotin is a synonym for the water-soluble B7-vitamin whereas streptavidin is a 53 kDa protein of bacterial origin. The biotin-streptavidin complex is one of the strongest known protein-ligand interactions with a dissociation constant Kd on the order of 10^{-15} M, i.e. the concentration of free biotin required to keep half of streptavidin molecules associated with biotin is no more than 10^{-15} M.
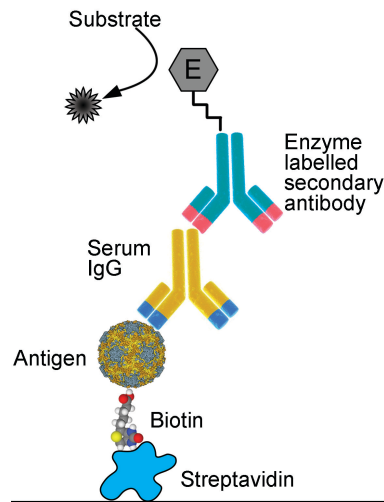
Figure 20. Schematic presentation of the IgG EIA format used in the thesis. Copyrights to the biotin, antigen and IgG images licensed from 123RF Ltd (Hong Kong, China).

To attach the biotinylated antigens to the streptavidin plates, the antigens were diluted in PBS + 0.05% polysorbate 20 (PBSP) and incubated on the plates for 60 min incubation at room temperature in a rocking (400 rpm) incubator. In Study III and Study IV 60 ng and 175 ng of antigen per well, respectively, was used. This significant increase in antigen load (to increase assay sensitivity) was probably made possible by the inclusion of low concentration of bovine serum albumin (BSA) in the antigen incubation buffer at 25 μg/ml.

The wells were blocked against unspecific antibody binding with proprietary blocking buffer ("sample diluent buffer"; Labsystems Diagnostics, Helsinki, Finland) containing proteins and detergents. Serum samples diluted 1:200 in PBSP were applied in 100 μl volume and incubated for 60 min at room temperature. The plates were washed three times (5 min each time) with PBSP and then incubated for one hour with horseradish peroxidase-conjugated rabbit anti-human IgG (DAKO, Glostrup, Denmark) diluted 1:2000 in the sample diluent. After four washes with PBSP, a mixture of orthophenylene diamine substrate and H_2O_2 was added. The reactions were terminated by adding 100 μl of 0.5 M H_2O_4 and absorbances were recorded at 492 nm after 10 min (Study III) or 15 min (Study IV).

IgM EIA without antibody absorption. IgM assays were done in μ-capture format as illustrated in Figure 21. Microtiter wells were first coated overnight at room temperature with 100 μl of goat anti-human IgM (Cappel/ICN

Biomedicals, CA, USA), diluted 1:500 in 0.05 M carbonate buffer (pH 9.6). Serum samples diluted 1:200 in PBSP and 0.05% PBSP were applied at 100 μl/well and incubated 60 min at room temperature. HBoV VLPs were applied at 25 ng/well and incubated 45 min at 37°C. The plates were then incubated 45 min at 37°C with 100 μl of horseradish peroxidase–conjugated streptavidin (Dako, Glostrup, Denmark) diluted 1:12000 in PBSP with 0.5% BSA. After each step, the sheet was washed 5 times with PBSP. Bound antigen was visualized by o-phenylenediamine dihydrochloride (Dako) substrate and H_2O_2 for 15 min at 37°C. The reactions were terminated by adding 100 μl of 0.5 M H_2O_4 and after 10 min absorbances were recorded at 492 nm.
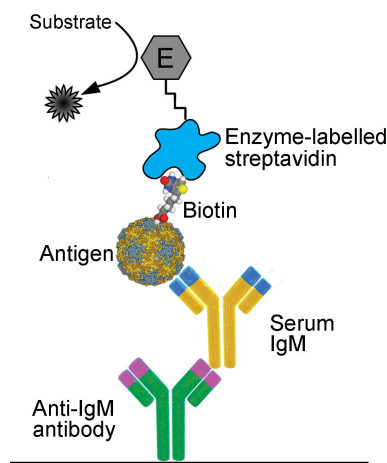
Figure 21. Schematic presentation of μ-capture IgM EIA. Copyrights to the biotin, antigen and IgG molecules licensed from 123RF Ltd (Hong Kong, China).

Introduction to antibody absorption. Measurement of species-specific anti-HBoV antibodies was accomplished with antibody absorption assays. As the name implies, these assays utilize a soluble antigen to bind and "inactivate" cross-reactive antibodies so that only specific antibodies can bind to the immobilized antigen. The assay principle is illustrated in Figure 22, with HBoV1 VLPs as the absorbing antigen and HBoV2 VLPs as the immobilized detection antigen.

Figure 22. Antibody-binding phase of HBoV2 EIA assays with and without HBoV1 absorption. VLP, virus-like particle; H1, HBoV1; H2, HBoV2; Ab, antibody. Copyrights to the biotin and IgG molecules have been licensed from 123RF Ltd (Hong Kong, China).
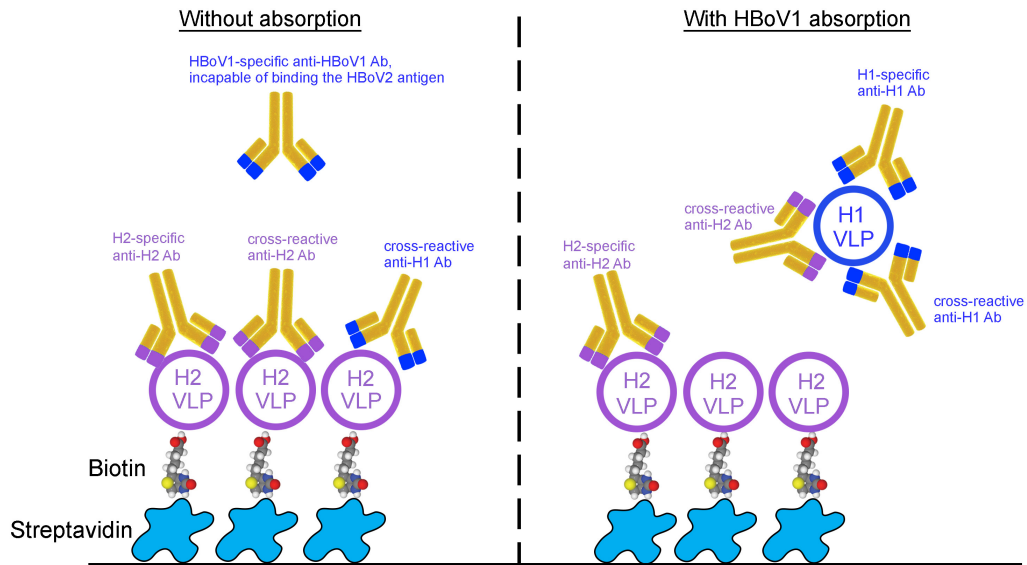
Antibody absorption in study III. The concentration of soluble VLPs required for exhaustive absorption of cross-reactive antibodies was tested in Study III by absorbing human sera with varying concentrations of homologous VLPs, and was found to be 30 μg/ml. Thus, for the detection of HBoV1-specific antibodies, we diluted the sera in PBSP containing soluble unbiotinylated HBoV2–4 VLPs (30 μg/mL each). After 1.5 h incubation at +4°C, the sera were applied on EIA wells coated with 60 ng of HBoV1 VLPs.

A converse approach was applied for the detection of HBoV2-4 whereby the sera were only absorbed with HBoV1 VLPs. Initial attempts to measure fully species-specific results by simultaneous absorptions with multiple VLP types reduced most absorbances below the detection level of our assay and were discontinued.

In all assays, the efficient absorption of the serum samples was assessed by testing the samples in parallel with competing VLPs that were homologous to the immobilized antigen. These residual optical densities (ODs) were typically very low (median 0.05; 90th percentile 0.12). Net ODs were calculated by subtracting the residual OD from the raw OD.

Other aspects of the EIA assays with antibody absorption (antigen amounts, incubation times etc) in Studies III and IV were as detailed above for the non-absorption assays.

Antibody absorption in study IV. HBoV1 absorption assays followed the absorption protocol used in study III. Preliminary HBoV2-4 IgG testing of all the children's follow-up samples also followed study III absorption protocol.

However, the increased sensitivity of EIA assays in Study IV (see above) enabled us to separate between HBoV2 and -3 –specific IgGs better than in the Study III. To distinguish between antibodies against these two viruses, seroconversion samples with HBoV2 and -3 IgGs in the initial quasi-specific screening (as well as the last follow-up sample of these children) were retested with species-specific assays. Specifically, the samples with HBoV2 and -3 IgGs in the initial screening were reanalyzed for HBoV2 IgG with simultaneous HBoV1 and -3 absorption. A similar double-absorption approach was used for the analysis of HBoV3 specific antibodies.

Testing of HBoV4 specific IgG in Study IV by HBoV1-3 absorption was limited to the middlemost and last follow-up sample of each DIPP child. This was based on the prior PCR data (chapter 5.2.5) and the results of Study III, both indicating that HBoV4 circulation among Finnish children is very rare.

For further details on the absorption protocols, please see Supplementary Figure 3.

Cutoff determination. In the absence of negative controls to HBoV2-4, the EIA cutoff in Study III were determined by a previously described inflection point analysis method [144], whereby absorbances from EIA measurements were plotted against the rank of each result. The analyses were based on the assumption that the ranked random values from uninfected individuals would roughly follow a straight line and an exponential deviation from this line at the inflection point would be a probable sign of exposure to the immunizing agent. The concept of inflection point, as defined in this thesis, is illustrated in Figure 23.
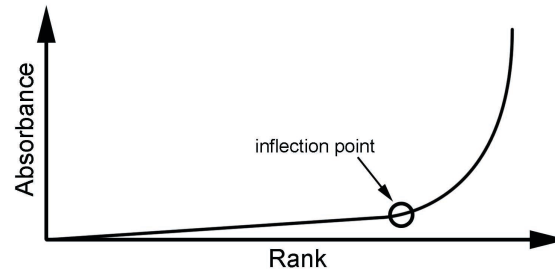
Figure 23. Visual representation of the inflection point as defined in this thesis; i.e. the starting point of exponential growth in an XY diagram.

However, it was later realized that linear correlation between ranks and absorbances was based on the false assumption of uniform absorbance distribution among the seronegative individuals. In reality, the data are likely to follow a normal or lognormal distribution. The interested reader can inspect the types of rank vs. absorbance curves that are obtained from a single population of lognormally distributed random data in Supplementary Figure 2. Furthermore the authors of the inflection point method [144] did not respond to an elucidation request, suggesting that the scientific validity of the method could be questionable. Fortunately, Study III deals largely with the cross-reactivity of HBoV1-4 antibodies, and the interpretation of the said data does not rely heavily on precise cutoff values.

The HBoV1-4 EIA cutoffs were re-established in Study IV by using serum samples from the cohort of children with genetic predisposition to type 1 diabetes (DIPP children; chapter 7.1.4). For IgG-EIA cutoff determination we selected samples (n=111) from children aged ≥1 years and before HBoV1 IgM seroconversion or HBoV1 viremia. We considered these samples to represent non-infected individuals without maternal IgG. Cutoff absorbances [mean + 4 standard deviations], for positive non-competed and competed IgG EIA results from these samples were 0.151 and 0.095, respectively. The same cutoffs were applied to the HBoV2-4 EIAs due to an inadequate number of control sera with HBoV2-4 IgM seroconversion or HBoV2-4 viremia.

For determination of the HBoV1 IgM-EIA cutoff, we selected serum samples (n = 138) from the DIPP-children aged >8 years on the basis that HBoV1 primary infections would be highly uncommon in this age group. Samples showing major fluctuations (≥ 0.5 absorbance units) in HBoV1-3 IgG absorbances at the sampling point or in neighboring follow-up samples were excluded. Cutoffs were calculated as the mean + 4 standard deviations of these

absorbances, yielding cutoffs of 0.131 and 0.092 for the respective non-competed and competed IgM assays.

8   RESULTS AND DISCUSSION

8.1   REAL-TIME QUANTITATIVE PCR DETECTION OF HBoV1-4 DNA (I)

Introduction. In Study I, real-time singleplex assays and a multiplex assay for the detection of HBoV1-4 DNA were developed, and their performance as diagnostic tools were also evaluated. The multiplex assay is still the only published method for the simultaneous detection of HBoV1-4 DNA and no other methods for the quantitation of HBoV3 or HBoV4 DNA have been published. Using this method, samples are first tested with initial multiplex assay, which gives a good estimate of the level of human bocavirus DNA. This preliminary screening is followed by singleplex qPCRs of the positive samples to identify and accurately quantify the amount of viral DNA.

Sensitivity. The sensitivity of a PCR assay is typically defined as the lowest quantity of template per reaction at which 95% of the positive samples are detected. These 95% detection limits were determined with serially diluted control plasmids. Specifically, testing was done by testing 8 replicates of 10^{-2} to 10^2 plasmid copies per reaction, diluted with pooled (n = 140) fecal DNA extracts (chapter 7.1.1) that were qPCR negative for HBoV DNA. The testing was repeated with another instrument on a different day. A generalized linear model was fitted to the data to determine the plasmid copy number that would yield a 95% probability of a positive result (Figure 24). Assay sensitivities in fecal DNA extracts ranged from ~4 copies per reaction (HBoV2/4) to ~9 copies per reaction (HBoV1) for both the multiplex and singleplex assays. The effect of human genomic DNA at concentration/amount of 500 ng/reaction on assay sensitivity was further assayed. At 10 copies/reaction, 100% of 20 replicates were positive with both the multiplex assay (regardless of HBoV species) and all singleplex assays. All in all, the assays have a robust limit of detection of ≤ 10 copies/reaction.
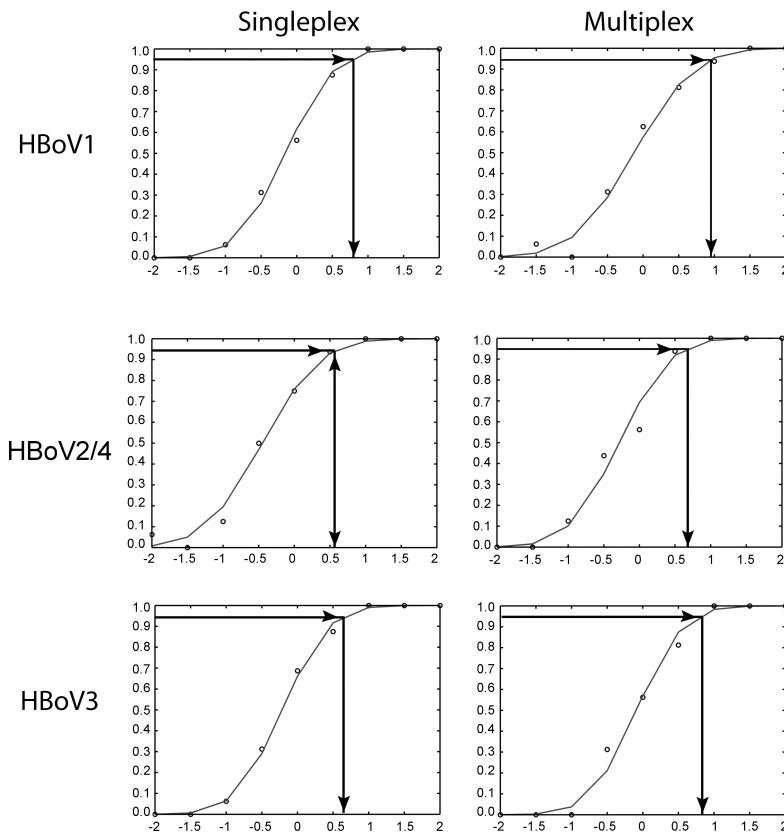
Figure 24. Analytical sensitivities of the real-time qPCR assays. The (log) number of plasmid copies is indicated on the X-axis and the proportion of PCR positive samples on the y-axis. The 95% detection probability level and the corresponding number of plasmid copies are indicated with arrows.

Specificity. The specificities of the qPCR assays were evaluated with cloned full-length or near-full-length genomes of Torque teno virus (TTV), parvovirus B19 genotypes 1-3, BKV, JCV and simian virus 40 at 10^{10} copies/reaction. Human genomic DNA purified from HeLa cells at 500 ng/reaction was also tested. Neither the multiplex nor the singleplex qPCR assays showed observable amplification with these specimens.

Cross-reactivity of the singleplex assays with HBoV1-4 templates was studied with 10^6 to 10^9 plasmid copies/reaction. No cross-amplification was observed with any of the assays at $\leq 10^7$ copies/reaction and the HBoV1 singleplex remained specific even at 10^9 copies/reaction. However, the HBoV2 and HBoV3 singleplex assays generated borderline positive signals at 10^9 and 10^8 copies of the converse templates, respectively.

Reproducibility. Reproducibilities of the multiplex and singleplex assays were studied by replicate analysis of quantification standards in a single run (intra-

75

assay variation) and repeated runs (interassay variation). The results are shown as coefficients of variation (COVs; ratio of standard deviation to the mean) in Study I in accordance with the commonly accepted guidelines for publication of quantitative real-time PCR experiments [145].

However, COVs by themselves are difficult to interpret. Comparison to other papers is also difficult because many papers incorrectly calculate interrun variation from quantification cycle data, which is subject to inherent interrun variation[vii]. To facilitate interpretation of assay variances, they are shown in Table 10 as averages of absolute deviations, defined as

$$\frac{1}{n}\sum_{i=1}^{n}|x_i - \bar{x}|$$

where x_i is the measured quantity of DNA and $\bar{x}$ is the mean of the three replicates.

The results show that the assays yield highly reproducible results. Even with only 10 nominal copies of template per reaction, the largest average deviation across all singleplex assays was only 6.5 copies/reaction.

[vii] Quantification cycle refers to the cycle number at which fluorescence has increased above the threshold. Random variation e.g. in the mastermix or the temperature of the halogen lamp used to excite the fluorescent molecules can cause a unidirectional shift in the quantification cycles of samples and controls. Since the former are determined from the latter, such unidirectional variation in cycle numbers is rather meaningless for the accuracy of DNA quantitation.

Table 10. Reproducibility of HBoV1, HBoV2/4 and HBoV3 singleplex assays as the average absolute deviation.

Copies per reaction	Intra-assay variation[a]			Inter-assay variation[b]		
	HBoV1	HBoV2/4	HBoV3	HBoV1	HBoV2/4	HBoV3
10^1	0.12×10^1	0.61×10^1	0.31×10^1	0.28×10^1	0.65×10^1	0.03×10^1
10^2	0.11×10^2	0.05×10^2	0.22×10^2	0.17×10^2	0.26×10^2	0.02×10^2
10^3	0.04×10^3	0.02×10^3	0.03×10^3	0.08×10^3	0.11×10^3	0.02×10^3
10^4	0.02×10^4	0.03×10^4	0.06×10^4	0.01×10^4	0.09×10^4	0.04×10^4
10^5	0.02×10^5	0.01×10^5	0.02×10^5	0.08×10^5	0.01×10^5	0.05×10^5
10^6	0.04×10^6	0.01×10^6	0.02×10^6	0.02×10^6	0.20×10^6	0.09×10^6
10^7	0.03×10^7	0.02×10^7	0.02×10^7	0.10×10^7	0.04×10^7	0.05×10^7
10^8	0.03×10^8	0.05×10^8	0.04×10^8	0.04×10^8	0.21×10^8	0.05×10^8

Note: average absolute deviation was calculated as $\frac{1}{n}\sum_{i=1}^{n}|x_i - \bar{x}|$ where $\bar{x}$ is the mean of the data set.

HBoV1-4 incidence in stool. For clinical evaluation of the new qPCR methods presented here, stool samples from 250 Finnish travelers and nontravelers (chapter 7.1.1) were screened for HBoV1-4 DNA. Five (2%) samples were found reproducibly positive for HBoV DNA in the initial multiplex test. Of these 5 samples, 4 and 1 were respectively positive with HBoV2 and HBoV3 singleplex assays, and positivity was further confirmed by sequencing. All four HBoV2 positive samples were from children aged <2 years, and the sample with HBoV3 DNA was from an 18-year-old individual. Although the absolute number of HBoV2-positive subjects was low, the relative HBoV2 prevalence was 12.5% among the 32 children aged below 10 years, and 20% among the 20 infants <2 years of age. Our results are therefore in line with previously reported incidences of HBoV2 DNA in stool (Table 4), supporting the specificity of the multiplex and (by extension) singleplex assays. Our results also reinforce the general consensus that HBoV2 DNA is detected mainly in the stools of children aged 5 years or less and rarely among adults. The limited age range of HBoV2 infections should be taken into consideration when interpreting the percentages of HBoV2-positive subjects.

Summary. Specific and highly sensitive real-time singleplex and multiplex assays were developed for the detection and quantification of HBoV1-4 DNA. All four assays reproducibly detected the genomic sequences of HBoV1-4 down to <10 copies/reaction. A single FAM labelled probe is utilized in all four singleplex assays and the multiplex assay. This minimizes technological

requirements for running the assays and enables the use of virtually all qPCR devices. The highly conserved probe sequence may furthermore provide a flexible and low-cost platform for the development of qPCR assays for additional, yet undiscovered bocaviruses. The results support the view that HBoV2 infections are relatively common in young children and rare among adults.

8.2 FIRST STEPS TOWARDS SEROLOGICAL DIAGNOSIS OF HBoV1 INFECTIONS (II)

Introduction. Diagnosis of acute HBoV1 infections relied initially on PCR analysis of respiratory samples. However, it quickly became clear that the line between acute and past infections is blurred by the extended presence of the virus in the respiratory tract of a significant proportion of infected individuals, even up to several months after initial infection [57,65,72,73]. Quantitative PCR and serum PCR alleviated the problem but did not entirely resolve it. This is because even high levels of HBoV1 DNA can persist for several months or recur in the respiratory tract of some children (chapter 5.2.4) and the potential brevity of viremia may limit its diagnostic value. PCR-based methods are furthermore poorly suited for assessing population-wide exposure to HBoV1-4.

To examine whether HBoV infections elicit systemic antibody responses and whether these responses could be used for the diagnosis of HBoV infections, we initially developed a western blot assay for the detection of anti-HBoV1 immune responses.

Serological diagnosis of HBoV1 infections. Paired serum samples from 117 children with acute wheezing were tested by immunoblotting using two recombinant HBoV capsid antigens: VP2 and the unique part of VP1 (VP1u). The sera were also quantitatively tested for HBoV1 DNA by others [56]. In this context, the concept of serodiagnosis is defined as presence of IgM and/or an IgG increase (including seroconversion). The subjective interpretation of immunoblot results was done before knowledge of the HBoV1 PCR results. The key findings in Study II were:

1) VP2 is superior to VP1u with respect to immunoreactivity. According to the VP2 assay, 51% and 28% of the 117 children had IgG or IgM, respectively. Corresponding VP1u-figures were only 7% and 2%. The following immunological results refer to reactivity against the VP2 antigen.

2) HBoV1 NPA load correlates well with serodiagnosis. Of 28 children with HBoV1 DNA load $<10^4$ copies/ml, 22 (79%) had serodiagnoses. In contrast, of the 21 of children with HBoV1 NPA DNA load $>10^4$ copies/ml, only 5 (24%) had serodiagnoses.

3) All 9 children with the strictest possible non-serological indicators of an acute HBoV1 infection (high NPA load, viremia and absence of other respiratory viruses) exhibited serodiagnosis.

4) Only 4 of 63 (6%) children without HBoV1 DNA in serum or NPA had a serodiagnosis.

The significance of these data for the diagnosis of acute HBoV1 infections by NPA PCR will be discussed in chapter 8.6. What is relevant here is how these data demonstrate that HBoV1 respiratory infections elicit B cell responses and that serological testing is an accurate tool for disclosing acute HBoV1 infections.

VP2's superior immunoreactivity in comparison to VP1u was surprising considering that VP1u of parvovirus B19 is very immunoreactive and has been successfully used in the diagnosis of B19 infections [146]. One potential explanation for this dissimilarity could be a difference in the location of VP1u within the capsid, influencing its accessibility to the host immune system [147].

In contrast to the dissimilar VP1u immunoreactivities, data in the Study II indicated that HBoV1 immunity resembles that of B19 with respect to the time-related conformational dependence of VP2 antigen recognition. B19 IgG responses during acute and early convalescence phases are directed towards both linear and conformational epitopes of VP2. However, antibodies against the linear epitopes disappear within 6 months and only the conformational antibodies persist [43]. Our immunoblotting results suggested that this same phenomenon affects HBoV1 immunity, since the HBoV1 VP2 IgG seroprevalence increased first with age, reaching a maximum of 52% among

children aged 1-2 years followed by decrease to 27% among children aged 3-5 years. The conformational dependence of antibody recognition was later supported by results obtained with conformational HBoV1 antigens (Study III), showing that HBoV1 IgG seroprevalence among children aged 1-2 years was indeed ~50% but, instead of decreasing, continued to increase relatively steadily to reach 77% in children aged 5-15 years. This difference in IgG seroprevalences between immunoblotting and conformational EIA is unlikely to be due to IgG waning and the better sensitivity of the latter method. As discussed in chapter 8.5, HBoV1 IgG levels are generally stable and appear to remain at a relatively high level after primary infection.

8.3 HBOV SEROLOGY REFINED (III)

Introduction. The western blot method used in the Study I provided the important first steps toward the establishment of serological diagnosis of HBoV infection. However, the laboriousness of the assay and its apparent inability to detect antibodies of past immunity prompted the development of more sophisticated EIA assays [113,137,148]. The combination of these IgG, IgM and IgG avidity methods, together with serum PCR, appeared to make HBoV1 diagnostics a straightforward endeavor. However, the discovery of HBoV2-4 in quick succession in 2008 [26] and 2009 [27] raised the concern that these closely related viruses could influence the diagnosis of HBoV1 infections. To characterize the extent of the serological cross-reactivity and to develop species-specific antibody assays for all four human bocavirus species, recombinant HBoV1-4 viral capsid proteins were produced in insect cells as virus-like particles (VLPs)

VLP expression. To produce recombinant antigens for EIAs, insect cells were infected with recombinant baculoviruses encoding the VP2 genes of HBoV1-4 viruses (Study III). With all four constructs, SDS-PAGE verified the high-level expression of ~60 kDa VP2. To analyze whether recombinant HBoV VP2s possess the capacity to spontaneously assemble to VLPs in insect cells, the proteins were purified by CsCl and examined by electron microscopy. Distinct parvovirus-like capsid structures with an approximate diameter of 21–25 nm

were observed with all four constructs as exemplified by HBoV2 VP2 VLPs in Figure 25.
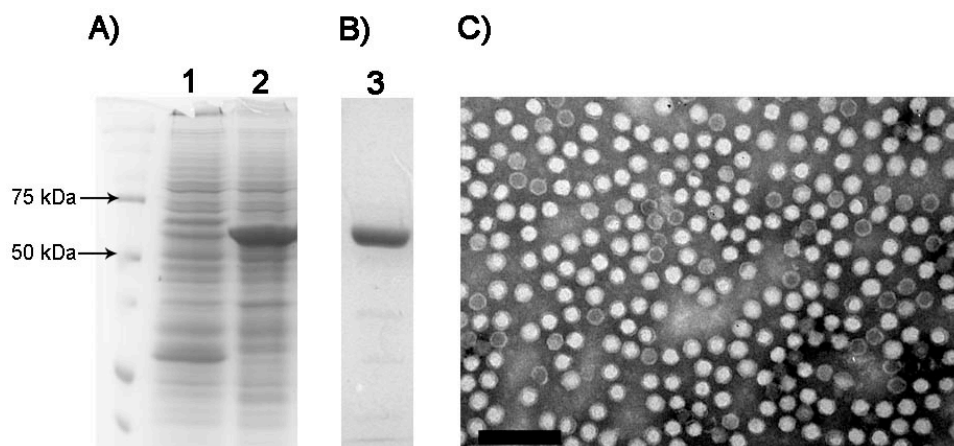

Figure 25. HBoV VP2 expression and purification with HBoV2 as a representative example. A) SDS-PAGE of non-infected (lane 1) and baculovirus-infected (lane 2) insect cells. B) SDS-PAGE of purified HBoV2 VP2. C) Electron micrograph of purified HBoV2 VLPs. Scale bar = 100 nm.

Antibody cross-reactivity. The VLPs were used in Study III to evaluate the cross-reactivity of anti-HBoV antibodies. The evaluation was done with human sera and with rabbit antisera produced against the HBoV1 or HBoV2 VLPs. The rabbit antisera were diluted serially and then tested by EIA (without absorption) for their reactivity to HBoV1-4 to determine the antibody endpoint titers. We also tested the antisera in a similar fashion against the VLPs of two other human parvoviruses and an animal bocavirus, namely human parvovirus B19, PARV4 and bovine parvovirus. Phylogenetically unrelated Merkel cell polyomavirus (MCPyV) VLPs were purified with the same method as the parvovirus VLPs and used as controls for unspecific binding.

Both HBoV1 and HBoV2 antisera showed clear cross-reactivity towards the other three bocaviruses in relative to the MCPyV negative control (Figure 26). Interspecies HBoV antibody titers were approximately 10^2-10^3 lower than homologous titers. Bovine parvovirus VLPs were unreactive with both antisera but surprisingly B19 and PARV4 VLPs showed modest reactivity. However, the almost linear shape of the B19 and PARV4 curves, as opposed to the inverse sigmoidal seen with HBoV antigens, may indicate random antibody binding rather than recognition of conserved epitopes.
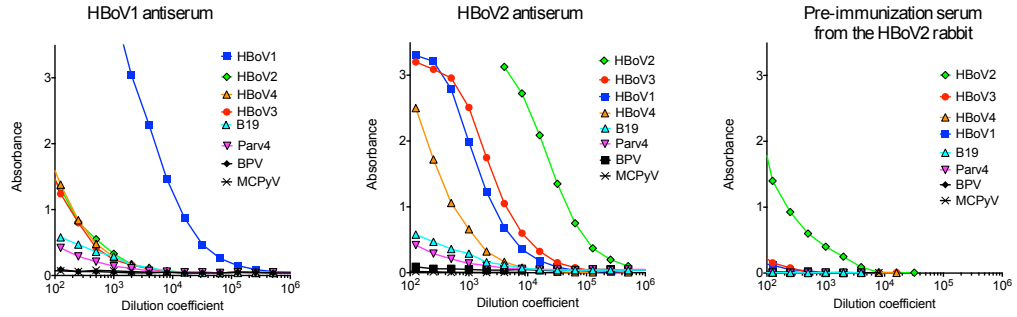
Figure 26. Endpoint dilutions of HBoV1 and HBoV2 rabbit antisera as indicated by the titles. The curve legends indicate the type of virus-like particle (VLP) that was used for antibody detection. The sera were studied with serial dilutions as indicated on the x-axis. Merkel cell polyomavirus (MCPyV) VLPs were used as a negative control to account for unspecific binding. The preimmune serum of the HBoV2 rabbit was the only preimmune sample to show antibody reactivity towards the VLPs before immunization and is also shown.

To assess the cross-reactivity of anti-HBoV antibodies in human sera among subjects with long-term immunity[viii], we measured the IgG reactivity of 115 serum samples from Finnish medical students with and without antibody absorption (Study III). The prominent effect of HBoV1 VLP absorption on the HBoV2-4 VLP reactivity of the sera is illustrated in Figure 27. The respective frequencies of HBoV2-4 IgG detection among the 115 adults were 96%, 87%, and 78% without absorption. HBoV1 absorption reduced these figures to 29%, 8% and 1%. HBoV1 seroprevalence and absorbance levels were affected more modestly by HBoV2-4 absorption, reducing the seroprevalence from 95% to 64%. In addition to significant cross-reactivity in human sera, the data illustrate that HBoV1 infections are significantly more frequent and/or elicit stronger antibody responses than HBoV2-4 infections. A more detailed discussion of HBoV1-4 seroprevalences will be found in chapter 8.5.

[viii] That the vast majority of these sera do in fact represent past-immunity phase of infection will be substantiated in chapter 8.5, showing that most individuals are infected by HBoV1 by the age of 5 years.
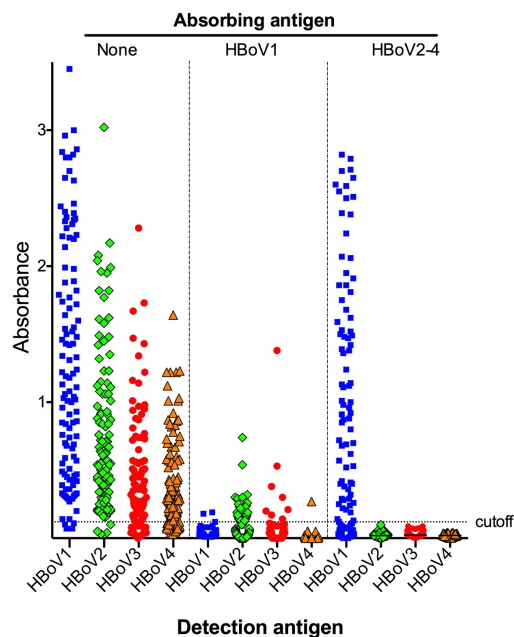
Figure 27. Immunoglobulin G (IgG) reactivity of 115 sera from Finnish medical students with HBoV1-4 virus-like particles (VLPs). Results are shown with and without antibody absorption as indicated by the top legends.

We also investigated the cross-reactivity of anti-HBoV IgG in individual medical students by monocompeting the serum samples with increasing concentrations of recombinant antigen (III). The sera were selected from individuals with IgG for only one bocavirus species. The results are illustrated in Figure 28. Neither human parvovirus B19 nor MCPyV absorption resulted in any observable reduction in absorbances, whereas absorption with heterologous HBoV antigens resulted in significant decrease in absorbance levels regardless of the underlying anti-HBoV IgG type of the seropositive individuals (Figure 28).
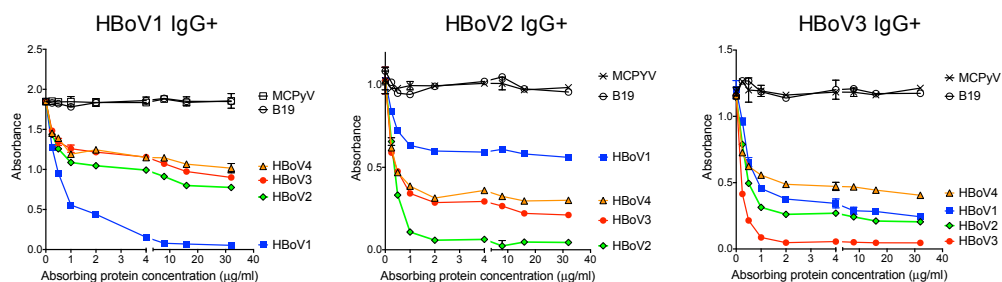
Figure 28. Monoabsorption of HBoV1, -2 or -3 IgG positive serum samples with various concentrations of homologous (colored curves) or heterologous (black curves) VLPs as indicated by the curve legends. The sera were selected from adults based on the presence of IgG for only one HBoV species as indicated by the image titles. The titles also indicate the type of VLP used for IgG detection. Merkel cell polyomavirus (MCPyV) VLPs were used to control for nonspecific competition.

To assess the cross-reactivity of acute-phase HBoV1 antibodies, we used paired sera from 21 wheezing children with a thoroughly characterized acute HBoV1 infection (II); all seroconverted for IgG, were viremic, and tested positive for IgM, and 17 (80%) also had HBoV1 DNA in NPA. Based on the results above, we expected these sera to show significant cross-reactivity in HBoV2-4 EIAs. However, to our surprise, we initially discovered that all these acute-phase sera were non-reactive in HBoV2-4 IgM EIAs and most were also unreactive in HBoV2-4 IgG EIAs (Figure 4A in Study III). Based on these initial results, it was suggested in Study III that antibodies against HBoV1 and possibly against HBoV2-4 are highly specific at the acute phase of infection but become more cross-reactive as the B cells mature and the affinity of the antibodies increases.

However, in Study IV the sensitivities of IgG and IgM EIAs were enhanced by increasing the amount of detection antigen and substrate incubation time as detailed in chapter 7.4.2. The increased sensitivity of the assays was counterbalanced by reduced interspecies HBoV specificity. With the enhanced IgG assay, sera from the children with acute HBoV1 infection were clearly reactive with HBoV2-4 VLPs (Figure 29; unpublished results), counteracting the previous claim of high specificity of acute-phase IgG (Study III). Similar results were obtained with the enhanced IgM assay. The previous proposition of highly specific HBoV antibodies in the acute phase of infection (as opposed to past-phase of infection) must therefore be rectified by acknowledging that the specificity depends heavily on the experimental methodology.
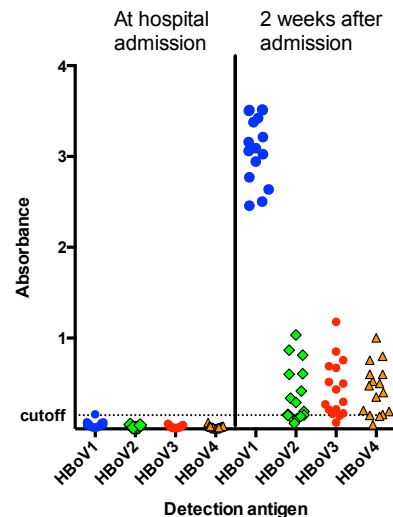
Figure 29. HBoV1-4 IgG reactivities of paired sera from 16 wheezing children (without antibody absorption) The sera were selected based on IgG seroconversion to HBoV1 VLPs, and most showed multiple markers of acute HBoV1 infection (IgM, viremia and HBoV1 DNA in NPA). This figure should be considered as an updated version of Figure 4A in Study III to reflect Study IV's improved assay sensitivities.

Since the results clearly demonstrate that anti-HBoV antibodies are cross-reactive, it is necessary to define concise symbols for results obtained without or with antibody absorption. Thus the subscript "a" (for "absorbed") will be used in the following chapters to denote the latter results, as in IgG_a or IgM_a. Absence of a subscript refers to results obtained without antibody absorption or, depending on the context, is a general reference to IgG or IgM antibodies.

Antibody absorption & EIA sensitivity. Antibody absorption assays block a significant portion of the total anti-HBoV antibody population and therefore reduce the sensitivity of the antibody assays (illustrated in Figure 22). This is demonstrated by Study III, in which it is showed that all 115 Finnish medical students were IgG positive for one or more HBoVs when measured without antibody absorption. Yet, in the competition assays, 16 (14%) individuals appeared negative for HBoV1-4. The situation improved by the development of the more sensitive EIA protocols (Study IV). By the new assays, only 2 of the 115 (1.7%) Finnish medical students were negative for HBoV1-4 IgG_a (unpublished results).

Despite this improvement in assay sensitivities, absorption assays consume very large quantities of antigens, which are laborious to produce and expensive to purify. Large-scale serological studies or routine inspection of anti-

HBoV antibodies calls i) for the development of EIAs that are not dependent on antibody absorption or ii) more advanced antigen production and purification methods. One future possibility is an EIA assay based on monoclonal species-specific HBoV1-4 antibodies (mAbs), which could then be competed with human serum antibodies. A reduction of absorbance in the mAb assay would indicate the presence of species-specific antibodies in the human serum. Another potential alternative for the identification and differentiation of acute HBoV1-4 infections is the screening of species-specific HBoV protein epitopes by epitope scanning. Indeed, a linear VP2 epitope suitable for the diagnosis of acute parvovirus B19 infection has been identified [149]. In Study II the groundwork for this kind of approach was laid by demonstrating that in linear form the HBoV1 VP2 antigen is highly reactive with acute-phase serum samples and superior in immunoreactivity to the unique region of the HBoV1 VP1 antigen.

Summary. As shown here and later confirmed by others [150], antibodies against HBoV1-3 show significant cross-reactivities, and correction for this is a prerequisite for VLP-based HBoV serology. Data regarding HBoV4 is scarce but there is no reason to assume that the virus would be antigenically distinct from the other HBoVs. Antibodies in acute-phase sera appear to be less cross-reactive than matured past-immunity antibodies but even acute-phase sera can cause false positive results in sufficiently sensitive HBoV antibody assays.

The cross-reactive antibodies can be blocked by antibody absorption but this reduces the sensitivity of the EIA assay. Antibody absorption also requires relatively high amounts of competing antigen and multiplies the number of analyses required for reliable results. For these reasons, new approaches should be considered for species-specific serodiagnosis of HBoV infections.

8.4 IMMUNOLOGICAL EFFECTS OF CONSECUTIVE HBoV INFECTIONS (IV)

Introduction. Significant cross-reactivities of anti-HBoV antibodies raised the possibility that antibody responses in consecutive heterologous HBoV infections could be influenced by the original antigenic sin phenomenon (OAS). As

discussed in chapter 5.3.1, OAS can be shortly defined as inhibited activation of naïve B or T cells toward a secondary antigen due to pre-existing immunity to a previously encountered (related) primary antigen. Such a non-optimal immune response could potentially lead to a more severe disease. Alternatively, the antigenic similarity of HBoV capsids could manifest as cross-protective immunity. We aimed to investigate the existence of these two phenomena by comparing the strength and frequency of HBoV primary infections to those of heterologous secondary infections.

To discuss the matter more concisely, the word "primed" will be used in this chapter to refer to children who had IgG$_a$ for one bocavirus before infection by *another* bocavirus. For example, the phrase "primed children with HBoV1 viremia" refers to children showing (persistent) HBoV2, -3 or -4 IgG$_a$ before showing HBoV1 viremia.

Codetection of IgG & IgM. Among the DIPP cohort, it was found that HBoV1 IgG$_a$ occurred more rarely in patients with HBoV2 IgG$_a$ and vice versa (IV). Specifically, all 51 HBoV2 IgG$_a$ negative subjects showed HBoV1 IgG$_a$ but only 43 of 58 (74%) HBoV2 IgG$_a$ positive subjects showed HBoV1 IgG$_a$ (p<0.0001 by the Fisher's exact test). When IgG$_a$ for two different bocaviruses were present simultaneously, reactivity toward the second virus was in general significantly lower than what was observed in non-primed individuals. For example, HBoV1 IgG$_a$ absorbance in all 68 non-primed children remained >1.0 after the initial seroconversion in contrast to only eight of 22 (36%) of children primed with HBoV2 or -3.

These results were observed with IgG assays that relied on antibody absorption. Since this technique may complicate the interpretation of the results, an HBoV1 IgM assay that does not require antibody absorption for high specificity (demonstrated in Study III, Figure 4B) was used to confirm the findings. It was found that (without antibody absorption) HBoV1 IgM occurred in 32 of 55 (58%) children in whom HBoV1 was the first infecting HBoV but only in one of 23 (4%) of children who were primed with HBoV2 or -3 before HBoV1 IgG seroconversion. The infrequent occurrence of secondary IgM in addition to secondary IgG in primed children is in line with the OAS phenomenon, since the production of both antibody classes originates from naïve B cell activation. Activated cells first secrete IgM but are later converted to IgG-secreting plasma cells through immunoglobulin class switching (reviewed

in [151]). Results from both the IgG and IgM assays therefore support the view that priming with HBoV2 or -3 decreases the likelihood of observing a subsequent seroconversion for HBoV1. Similarly, priming with HBoV1 decreases the likelihood of observing a subsequent seroconversion for HBoV2 or -3. Low frequency of secondary (heterologous) seroconversions may also reflect cross-protective immunity e.g. via cross-neutralizing antibodies.

Viremia. The above results demonstrate that priming can significantly reduce antibody responses against novel epitopes of another bocavirus species or protect against heterologous infections. However, they do not tell if priming has the potential to completely prevent serological (species-specific) identification of secondary infections if one has indeed taken place. To gain further insight into immunological aspects of secondary bocavirus infections, it is essential to inspect a diagnostic marker that does not depend on the activation of naïve B cells, is unlikely to be caused by aerial contamination and is a highly probable sign of an acute infection: viremia.

Among the DIPP cohort, five primed children with HBoV1 viremia and four primed children with HBoV2 viremia were identified. Two of the five HBoV1 viremias and all four HBoV2 viremias failed to result in a detectable IgG_a response specific for the viremic species. IgG_a responses that did take place against the viremic species were generally much weaker than in non-primed children. To illustrate these results, the complete IgG profiles (i.e. with and without antibody absorption) of two primed children (#122, #114) with HBoV1 or HBoV2 viremia are shown in Figure 30. Child 122 showed HBoV1 viremia at the age of 4 years without developing HBoV1 IgG_a, whereas child 114 showed HBoV2 viremia slightly before the age of 8 years and developed only a borderline HBoV2 IgG_a response. The complete IgG profiles of two representative non-primed viremic children (#53, #31) are shown for comparison. The interested reader can also find as an online supplementary data the IgG profiles of all 109 children of the DIPP cohort using the digital object identifier[ix] (DOI) 10.6070/H4ST7MRT. The IgG profiles of the three children who were primed with HBoV2 but managed to generate an antibody response against a viremic HBoV1 infection appear in the supplementary file as cases #15, #36 and #69.

[ix] The DOI code can be translated into a web address at dx.doi.org. For more information on DOI codes, please see the Foreword (chapter 4).
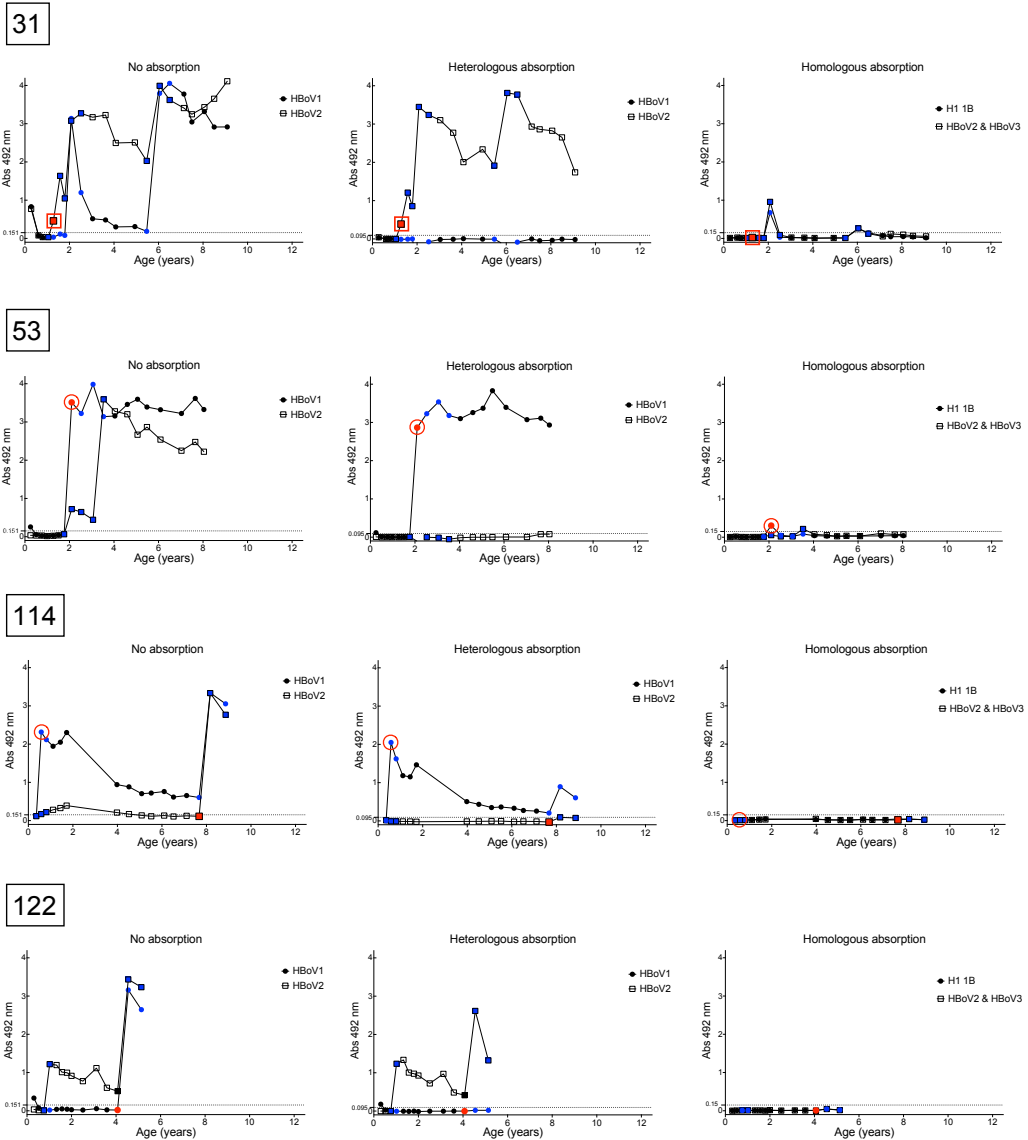
Figure 30. Representative IgG profiles of two primed (114, 122) and non-primed (31, 53) children with HBoV1 or HBoV2 viremia (for the definition of the word "primed" as used here, please see the introduction paragraph of the current chapter). There are three graphs for each subject and a row corresponds to one subject. The black, blue and red data points respectively indicate whether the serum has not been tested by PCR, has been negative by PCR or has been tested positive by PCR. Red open circles and rectangles respectively indicate that the sample has been IgM positive for HBoV1 or HBoV2. Heterologous absorption (middle graphs) here means that HBoV1 IgG$_a$ measurement has been done with HBoV2 and -3 absorption whereas HBoV2 IgG$_a$ measurement has been done with HBoV1 absorption. The rightmost graphs are from homologous absorption (i.e. same antigen used for measurement and absorption) to control the thoroughness of the absorption. Note that these children tested negative for HBoV3 IgG$_a$. For more information on the absorption procedures, please see chapter 7.4.2.

As an alternative to the OAS interpretation, one could perhaps argue that not all HBoV viremias result in IgG$_a$ responses regardless of the preexistence of antibodies towards other bocavirus species. In the case of HBoV1, this seems

very unlikely considering that all the documented HBoV1 viremias in non-primed children were associated with very strong IgG_a responses, as exemplified by child 53 in Figure 30. The only exception is the child #65 (see the online supplement) but this individual had maternal HBoV1 IgG_a during the viremic period. IgG_a responses to viremic HBoV2 or HBoV3 infections in unprimed individuals were generally weaker than those against HBoV1, but hardly weak enough to support non-immunogenic viremias in unprimed individuals.

The results point to a remarkable similarity between the serodiagnostic aspects of HBoV and dengue virus (DENV) infections. The identity of the priming DENV serotype can be determined by measuring which serotype the patient's serum sample neutralizes most efficiently. In line with OAS, the highest titer is against the priming serotype even after infection by other serotypes [152]. However, despite a few decades of research, there is no proven serologic method to reliably determine the serotype responsible for a secondary DENV infection. One recent study [153] used a multinomial logistic regression model to infer the secondary infecting serotype with 68% accuracy based on the patient's pre- and postinfection neutralizing antibody titers (the true serotype had been determined by blood PCR). The model inferred the infecting serotype correctly in 60% of cases if only the postinfection neutralization titer was known (as would be the case in most clinical settings).

Analogously to neutralizing antibody titers against dengue viruses, a secondary bocavirus infection could potentially in the future be identified by comparing pre- and postinfection antibody titers against shared and specific HBoV epitopes. A homologous reinfection should efficiently stimulate the generation of IgG *and* IgG_a against the priming species whereas one might expect a heterologous infection to result only in the generation of antibodies against epitopes that are shared with the priming bocavirus species. Furthermore, as indicated by the regression model used to type DENV infections, relative increases in antibody titers towards the cross-reacting epitopes could identify the secondary species involved in the infection.

However, as indicated by the ambiguousness of DENV serology, precise typing of the secondary infections could be impossible. Even serological separation between homologous and heterologous secondary infections by comparing changes in IgG and IgG_a titers could prove very challenging. This is due to the possibility that the second HBoV species may stimulate B-memory cells specific for the first HBoV species. Whilst such an effect may seem

paradoxical, it has been observed as early as 1974 by Virelizier and colleagues who examined immune responses against purified H_0 and H_1 hemagglutinin, representing two influenza strains [121]. The authors suggested two possible explanations:

> *"Either T-memory lymphocytes, with a broader specificity than the B lymphocytes, are triggered by the cross-reacting antigen and are then able to help B-memory lymphocytes to secrete antibody specific for the first antigen, as suggested in other systems, or B-memory cells with specificity for the first antigen are directly triggered by the cross-reacting antigen. Our results favor the latter hypothesis, since H_0-primed spleen cells transferred after treatment with anti-θ serum[x] and complement were able to secrete anti-H_0 antibody in either irradiated or thymus-deprived recipients after a boost with H_1. "*

Child #114 in Figure 30 could be example of this type of phenomenon. At the age of 8 years, one can see HBoV2 viremia followed by borderline HBoV2 IgG_a response and a clearly stronger HBoV1 IgG_a response. Simultaneously, the child shows a very strong rise in IgG against epitopes shared between HBoV1 and HBoV2. The absolute number of similar cases in the DIPP cohort cannot be determined because the EIA absorbances in many children were already near the saturation point (Abs > 3.0) of our assays. Proportional estimates can nevertheless be made by examining children with absorbance values under saturation level (<3.0) before the secondary heterologous IgG_a seroconversion. Of 28 such children, 11 (40%) showed a coincident increase in IgG_a (ΔAbs ≥1.0) against the priming bocavirus species.

Due to the implications of OAS, the interpretation of the serological data is challenging and the possibility of heterologous IgG_a boosts complicates it even further. Let us examine e.g. the child #115 (Figure 31). At the age of ~6 years, this child showed a sudden very sharp increase in non-absorbed IgG towards HBoV2 and HBoV3 and a simultaneous increase in IgG_a towards HBoV1. If one were to set aside heterologous IgG_a boosts, the secondary increase in HBoV1 IgG_a would be interpreted as an obvious sign of a re-exposure to HBoV1. However, the notion of heterologous boosting and a comparison of the IgG and IgG_a absorbances in the two figures offers an alternative

---

[x] anti-θ serum contains cytolytic antibodies against T-cells

interpretation. Before the antibody boost, the IgG and IgG$_a$ curves show a very good correlation with the latter showing only slightly lower absorbance values than the former. After the antibody boost, HBoV1 IgG$_a$ absorbance quickly decreases back to the pre-boost level but the non-absorbed IgG absorbance remains at very high level with all three antigens. This change is especially noteworthy regarding the HBoV2 and -3 IgG absorbances that were near cutoff-level before the boosting event. Thus, this case could be an example of strong heterologous IgG$_a$ boost combined with the OAS phenomenon. Although strongly speculative, this demonstrates the complexity of untangling the serological data.
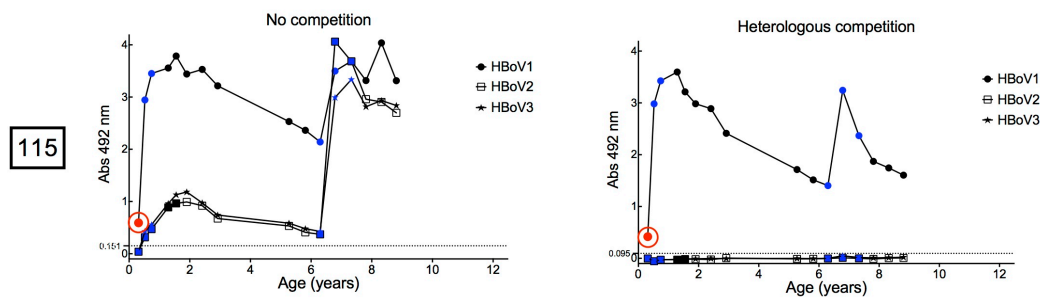

Figure 31. Speculative example of a heterologous HBoV IgG$_a$ boost by an enteric bocavirus. See Figure 30 footnote for descriptions of the symbols.

OAS vs. cross-immunity. The term "original antigenic sin" is wordplay that refers to the Christian theological doctrine of Adam and Eve tasting the forbidden fruit in Eden, and the consequences of this incident[xi]. As already discussed in chapter 5.3.3, the phenomenon has at least theoretical potential to result in aggravated viral infections. However, the negative undertone of the term may not necessarily be justified in the context of human bocaviruses. This is because protective aspects of HBoV immunity could explain most manifestations of the phenomenon that we have seen in the preceding chapters. As already discussed in chapter 5.3.2, preexisting immunity against the priming bocavirus species may significantly limit the capability of the secondary species to propagate by eliminating infected cells with cytotoxic T cells targeting conserved epitopes. Second, some of the cross-reactive antibodies may directly

---

[xi] Genesis 3:16-19: Then God said to the woman, "I will sharpen the pain of your pregnancy, and in pain you will give birth. And you will desire to control your husband, but he will rule over you". To Adam he said, "Because you listened to your wife and ate fruit from the forbidden three, cursed is the ground under your feet; through painful toil you will eat food from it all the days of your life. It will produce thorns and thistles for you, and you will eat the plants of the field. By the sweat of your brow you will eat your food until you return to the ground; for dust you are and to dust you will return."

neutralize the secondary virus or facilitate its elimination through phagocytosis. Even in the absence of robust responses against the unique epitopes of the secondary bocavirus, together these factors and the innate immunity could potentially prevent secondary HBoV infections entirely or reduce the incidence of systemic infections by preventing the spread of the virus.

The protective or exposing effect of HBoV priming could potentially be examined by comparing the occurrence and severity of symptoms associated with primary infections and secondary heterologous infections. However, the number of children with secondary HBoV seroconversions was insufficient for meaningful statistical analysis. Symptoms associated with primary HBoV infections will be discussed in chapter 8.7.

One could also attempt to compare the magnitudes and frequencies of viremias in primary and secondary infections. However, this was complicated by the non-constant sampling intervals, which were doubled beyond 2 years of age. Since the likelihood of viremia detection is directly proportional to the frequency of sampling and children with primary infections were on average younger than children with secondary infections, the rates of viremias in the two groups cannot be directly compared. Age matching was attempted to circumvent this problem but the number of matched children was insufficient for a meaningful statistical analysis.

Summary. Pre-existing immunity against HBoV1 is associated with weak or non-detectable immune responses against the following HBoV2 infections and vice versa. This suggests that close antigenic similarity of HBoV1-4 gives rise to the OAS phenomenon, cross-protective immunity or both. However, the absence of detectable IgG_a responses to viremic infections in some primed individuals suggests that the results are not only due to cross-protective immunity preventing secondary infections. These observations have important implications for the interpretation of serological HBoV data and emphasize how the accurate diagnostics of HBoV1 and -2 (and possibly -3 and -4) infections calls for the parallel use of nucleic acid and serodiagnostic methods. Future serodiagnostic methods to identify secondary HBoV infections could potentially be based on the relative strengths of antibody responses against species-specific and shared HBoV epitopes.

8.5   HBoV1-4 IgG SEROPREVALENCE AND LONGEVITY (IV)

Introduction. Since the discovery of HBoV1 in 2005, several serological surveys have assessed its seroprevalence [148,154-158]. These studies have uniformly reported that HBoV1 seroconversions occur early in life and that HBoV1 IgG is found in almost all adult individuals. However, this thesis project is the first to assess HBoV1 seroprevalence with species-specific antibody assays that take into account the cross-reactivity between HBoV1-4. This is also the first assessment of HBoV2-4 seroprevalences among adults and children.

Seroprevalence. In Study IV in which 109 children were followed almost from birth to early teens by sequential serum sampling, all children became seropositive for one or more HBoV species by the age of 4.8 years. The median ages of HBoV1-3 seroconversions were 1.9, 1.7 and 1.6 years, respectively, with no statistically significant difference. The most commonly detected IgG_a seroconversions were, in descending order, against HBoV1, -2 and -3. HBoV4 IgG_a was not detected in any of the children. Frequency distributions of HBoV1-3 seroconversions in various age groups are illustrated in Figure 32. The cumulative and non-cumulative seroconversion frequency distributions in the graph differ from each other because some of the children reverted to seronegative status. HBoV1-3 IgG_a seroreversions were evident in 10 (11%), 13 (22%) and 3 (30%) of the seroconverted children, respectively. Approximately half of the IgG_a seroreversions occurred in children with very low-level IgG_a responses that lasted only one or two sampling intervals (e.g. child #110 in the supplementary data). The other half occurred in children with weak or moderate IgG_a responses that waned below the cutoff level over the course of a few years (e.g. child #14 in the supplementary data).
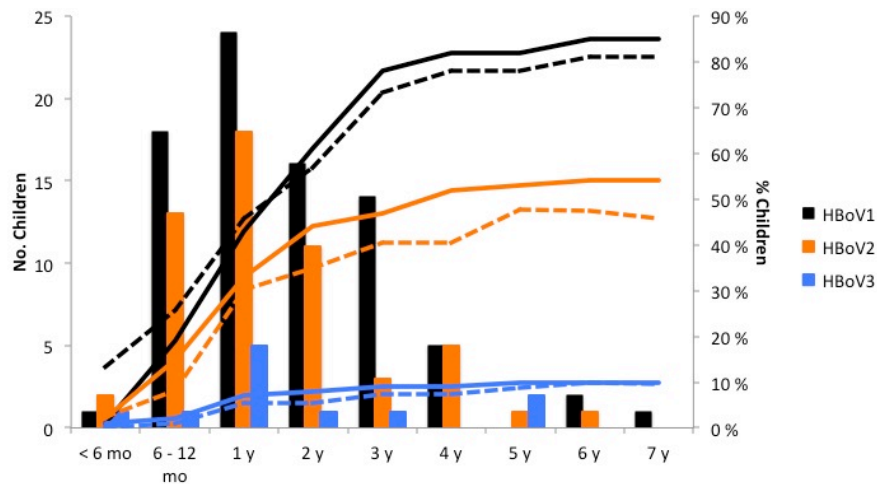
Figure 32. Cumulative (lines) and non-cumulative (blocks) frequency distribution of human bocavirus IgG$_a$ seroconversions in different age groups. Seroprevalences are shown with dashed lines.

The reader is cautioned that, due to the probable influence of OAS, the true incidence of HBoV1-3 infections is likely to be higher than that indicated by the seroprevalence figures. It is also possible that the HBoV2-4 EIA assays are not sensitive enough, since the protocols are based on the HBoV1 EIA assays and not individually optimized for HBoV2-4 IgG detection (discussed in the end of chapter 7.4.2). The figures shown herein should therefore be considered minimum estimates of the incidence of HBoV1-3 infections in Finland. For comparison, a Chinese study [150] examined HBoV seroprevalences in different age groups and found that approximately 60%, 50%, 40% and 0% of children aged 5-14 years were positive for HBoV1-4 specific antibodies, respectively. The most significant differences to the results presented in this thesis are the low HBoV1 IgG$_a$ seroprevalence and high HBoV3 IgG$_a$ seroprevalence. This could indicate that there are significant regional differences in the relative incidences of HBoV1-3 infections. However, PCR data does not appear to support the wide circulation of HBoV3 in China. Studies assessing the incidence of HBoV DNAs in Chinese stool specimens [100,106] have been consistent with corresponding western studies (chapter 5.2.5), showing that HBoV3 DNA is found very infrequently.

Our studies III and IV and this Chinese study, published 9 months after Study III, are currently the only published analyses of HBoV1-4 seroepidemiology that have taken into account serological cross-reactivities between HBoVs.

IgG longevity. IgG longevity varied greatly between individual children, but generally HBoV1 IgG$_a$ profiles were characterized by strong initial seroconversions that remained at high level throughout the sampling period (e.g. child 53 in Figure 30). HBoV2 IgG$_a$ profiles were characterized by sharp increases and decreases in absorbance and generally the absorbance levels were lower than those of HBoV1 (e.g. child 122 in Figure 30). The number of HBoV3 positive subjects was too low for firm conclusions to be drawn but the IgG$_a$ profiles of the few seropositive children resembled those of HBoV2.

Weak and/or waning IgG responses to HBoV2 and HBoV3 are supported by the low IgG$_a$ levels among the Finnish medical students (Study III; Figure 27). Of note is also the finding that maternal IgG$_a$ against HBoV1 was detected in 51% of 88 DIPP children from whom the first serum sample was taken within 6 months from birth (Study IV). Maternal IgG$_a$ against HBoV2 or HBoV3 occurred only in 7% of this same group of children, further indicating low antibody levels in adult women. Distinction between maternal HBoV2 and HBoV3 IgG$_a$ was not possible in absorption assays due to low antibody levels.

Summary. Serological results support PCR-based findings that the HBoV species most frequently infecting humans are, in descending order, HBoV1, HBoV2, HBoV3 and HBoV4. HBoV1-3 infections occur early in childhood. There was significant variability in IgG$_a$ stability between individual children but typically HBoV1 IgG$_a$ levels remained high and stable, whereas HBoV2 IgG$_a$ levels were markedly lower and characterized by sharp fluctuations. These data, together with the infrequent occurrence of HBoV2 viremia among children (next chapter), may indicate that the enteric bocaviruses typically cause local infections of the intestinal tract rather than systemic infections like HBoV1.

8.6 HBoV DNA AS AN INFECTION MARKER (II, IV)

HBoV1 DNA in respiratory samples. The week or so required for the primary adaptive immune response to develop against an infecting pathogen is the time when the body is especially vulnerable and the clinical symptoms of the infection can typically be expected to manifest. To assess the pathogenicity of a novel virus, it is important to know how well a diagnostic test can distinguish the acute phase of infection from the recovering (convalescent) or past phase of

infection. Inability to distinguish between the phases could significantly reduce the correlation between infection and disease.

To assess the diagnostic significance of finding HBoV1 DNA in nasopharyngeal aspirates (NPAs), NPA PCR positivity was compared to the occurrence of HBoV1 IgM and IgG in paired serum samples from 117 children with acute wheezing (Study II). The first of the two serum samples was obtained at hospital admission (acute sample) and the second sample 2-3 weeks later (convalescent sample). A serodiagnostic result was defined as the presence of IgM in either of the two consecutive samples or a significant increase in IgG between the two samples.

As discussed in chapter 8.2, 24 of the 28 (96%) wheezing children with high HBoV1 viral NPA load showed HBoV1 serodiagnosis (Study II). In contrast, only five of 21 (24%) children with low viral NPA load showed HBoV1 serodiagnosis. Allander and colleagues had previously tested most of these same sera for HBoV1 DNA and found 94% of children with high NPA load to have HBoV1 DNA in acute serum as opposed to only 11% of children with low load [56]. Together these results are consistent with the presence of relatively high NPA load at the acute phase of infection and with subsequent low-level persistence. Indeed, several later studies have confirmed that HBoV1 DNA may persist or reappear in respiratory airways for up to several months after primary infection [57,65,72,73]. For example, a Finnish study [72] found that 11% of 152 immunocompetent children showed prolonged (or intermittent) presence of HBoV DNA for at least 3 months whereas a Danish prospective birth cohort study [65] found that 25% of immunocompetent children remained positive for HBoV DNA for at least 2 months. The poor diagnostic value of qualitative HBoV1 NPA PCR may be further exacerbated by the frequent exposure of children to HBoV1 DNA by their infected peers, e.g. in day care. The extreme sensitivity of modern DNA detection methods makes it possible that inhalation of exogenous HBoV1 DNA can yield a positive PCR result without causing infection in a seropositive individual. It is also possible that low-level HBoV1 DNA in the respiratory tract is indicative of a superficial or a very localized infection without systemic immunization. If this indeed is the case, such superficial infections are unlikely to result in significant symptoms and would further reduce the usefulness of non-quantitative DNA assays in HBoV1 diagnostics.

However, even high HBoV1 NPA load is not a definite marker of recent infection. This has been demonstrated in two relatively recent studies. Lehtoranta and colleagues [72] showed that ~10% of 152 otitis-prone children exhibited prolonged presence of HBoV DNA for ≤3 months. Of these prolonged cases, approximately half had respiratory viral loads that remained above 10^4 copies/ml. Furthermore, Martin and colleagues [57] observed in 3 out of 70 HBoV1 DNA-positive (constitutionally healthy) children viral loads of 10^6 copies/ml more than a month after initial PCR positivity.

Prologue to HBoV viremias. Because of the discovery of HBoV1 from nasopharyngeal aspirates and HBoV2-4 from fecal samples most studies have assessed the involvement of HBoV1 in respiratory and HBoV2-4 in gastrointestinal disease. However, viremic (or "systemic") infections result in the distribution of the viruses throughout the body and this may give rise to symptoms of other types. Understanding the tendency of HBoV1-4 to cause viremia therefore facilitates the assessment of their possible etiological roles. On the other hand, the duration of viremia determines its diagnostic value in the identification of acute HBoV infections.

The incidence and duration of HBoV1-4 viremias were analyzed in the wheezing children (studies II & III) and the DIPP children (Study IV). Most serum samples from the wheezing children were analyzed for HBoV1 DNA in studies that are not part of this thesis [56,113] but testing for HBoV2-4 DNA was done as part of Study III. For the PCR study of the DIPP sera, we devised a serological selection method to limit the testing to 564 samples of the entire set of 1943 samples. Specifically, quantitative PCR was done on the three specimens flanking each primary or secondary increase (>1.0 absorbance units) in anti-HBoV1-3 antibodies as illustrated in Figure 33.
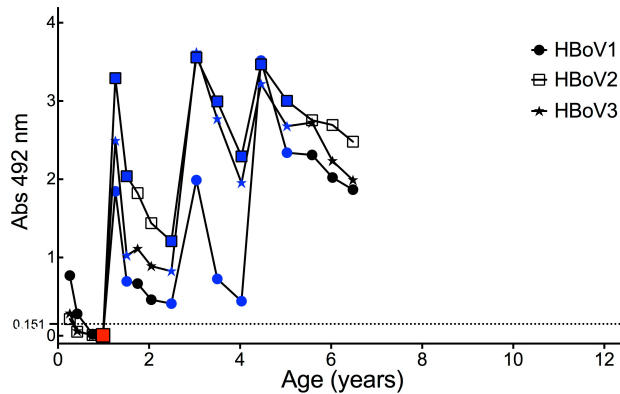
Figure 33. Sample selection for studying the incidence of viremia in the DIPP cohort was based on IgG fluctuations. The image illustrates (without absorption of cross-reactive antibodies) the HBoV1-3 IgG absorbances of child #52 followed ~7 years from birth. All samples showing an >1 absorbance unit increase in IgG for any of the three viruses were included in the screening, as well as samples flanking these increases. PCR negativity and positivity are indicated by blue and red colors, respectively. Samples that did not meet the inclusion criteria are shown with black symbols.

HBoV1 viremias. Among the 117 wheezing children, diagnostic HBoV1 antibody results coincided almost invariably with HBoV1 viremia. Of the 29 children with an HBoV1 primary infection according to immunoblot analysis, 27 (93%) were viremic (Study II). An extended cohort of 258 wheezing children that included these 117 subjects was later analyzed by HBoV1 VLP EIA. This study showed a very similar result; of 48 children with serologically diagnosed acute HBoV1 infection, 45 (94%) were viremic. On the other hand, as mentioned earlier in this chapter, a study by Allander and colleagues [56] on the full cohort of 258 wheezing children showed that 94% of the children with a high respiratory viral load ($\geq 10^4$ copies/ml) were viremic as opposed to only 11% of children with low load ($< 10^4$ copies/ml). Together these associations indicate that HBoV1 viremia is a good marker of recent and, due to its association with high NPA loads, clinically most relevant HBoV1 infections.

However, these results do not necessarily mean that typical HBoV1 primary infections are associated with viremia. The patients were enrolled based on hospitalizing respiratory symptoms and therefore represent the more severe spectrum of HBoV1 infections. The DIPP cohort provides a better model of typical HBoV1 infections among children but the long sampling interval of 2-6 months inevitably downplays the incidence of viremias. Of the 94 DIPP children who demonstrated HBoV1 IgG$_a$, 23 (24%) were found to be viremic. Viremias coincided almost invariably with HBoV1 IgG$_a$ and/or IgM$_a$ seroconversion; the few exceptions have been discussed in chapter 8.4.

The length of a typical HBoV1 viremia can be roughly approximated by combining the data of studies II-IV. According to the DIPP results (IV), none of the 24 children with viremia showed HBoV1 DNA in the follow-up sample taken 3 to 6 months later. Assuming that these 24 individuals are representative of the general population, this yields an upper limit for the duration of viremia for most immunocompetent children. On the other hand, only 10 of the 44 (23%) wheezing children with HBoV1 viremia at the time of admission were still viremic 2-3 weeks after discharge (Study II; time at the hospital was 1-3 days). If it was assumed that the onset of viremia preceded hospitalization by a few days, then in most individuals HBoV1 viremia would be expected to last less than 3 weeks. If no association was assumed between HBoV1 and the onset of the wheezing symptoms, then on average the first sampling could be expected to have taken place in the middle of the viremic period. This means that, regardless of the association of HBoV1 with respiratory symptoms, in most individuals HBoV1 viremia lasts less than 6 weeks.

Contrasted with the observations made in the Study IV on the exclusive occurrence of HBoV1 viremias in primary infections and in early childhood, a study by Bonvicini and colleagues described the presence of HBoV1 in 6% of healthy adult blood donors [159]. Long persistence of HBoV1 DNA in blood would be in line with the kinetics of human parvovirus B19 viremia, which may persist for months and possibly even years in the blood of immunocompetent individuals [160,161]. However, Bonvicini and colleagues relied on a fluorescent DNA binding dye as the PCR product identification method. This method by itself is much more prone to false positive results than qPCRs based on hydrolysis probes, since SYBR green reacts with any double stranded DNA and the specificity of the assay relies solely on specific primer binding. Furthermore, the authors did not disclose any method (e.g. sequencing or melting point analysis) to verify their results.

HBoV2-4 viremias. None of the 248 wheezing children were viremic for HBoV2-4 as observed in Study III. However, this needs to be put in the context of few acute HBoV2-4 infections among these children. Only 5 (2%) of the 248 children showed an IgG seroconversion for HBoV2, 2 (0.8%) for HBoV3 and one (0.4%) for HBoV4. For comparison, 13% of these children showed an IgG seroconversion for HBoV1 and 19% [113] had HBoV1 viremia. Incidentally, these

data show that HBoV2-4 do not have a significant role in acute expiratory wheezing in Finland.

In the entire DIPP cohort of 109 children, 59 (54%) showed HBoV2 IgG seroconversion and 11 (10%) HBoV3 seroconversion. Only 3 (5%) of the children with HBoV2 seroconversion showed HBoV2 viremia (all coinciding with the HBoV2 seroconversion). One child of the 11 HBoV3 seroconversions (9%) had HBoV3 viremia. Viral loads were $<10^4$ copies/ml in all cases. As discussed in chapter 8.4, four HBoV2 viremias were also discovered in HBoV1 seropositive children that did not result in a detectable IgG_a response against the viremic species. The concentrations of viral DNA in the sera of these children were also low, $<10^4$ copies/ml.

In addition to studies III and IV, as outlined here, only two relatively small-scale studies addressing the occurrence of HBoV2-4 viremias have been published. Mitui and colleagues tested 79 serum samples from an equal number of children with encephalitis [162] and found HBoV2 DNA in one serum (with no other pathogen detected). Paloniemi and colleagues tested serum samples from six children with gastroenteritis and HBoV2 DNA in stool, and found viremia in two sera [163]. Neither study reported the quantity of viral DNA in the sera.

Summary. HBoV1 viremia is a reliable marker of a very recent or acute HBoV1 infection. HBoV1 viremia is also associated with IgG/IgM seroconversions and high NPA load. This and the seclusion of blood circulation from exogenous (contaminating) DNA makes viremia a good marker for diagnosing HBoV1 infections. In contrast, HBoV2 viremias appear to be rare and to coincide infrequently with HBoV2 seroconversion. As discussed in the previous chapter, this may indicate that the enteric bocaviruses typically cause localized infections of the gastrointestinal tract and not systemic infections like HBoV1.

8.7 CLINICAL CORRELATES OF HBOV INFECTION (IV)

The scheduled sampling of the 109 DIPP children in study IV included an interview of the parents by a study nurse about any clinical symptoms or illnesses since the previous visit. This enabled us to compare symptom

frequencies in the HBoV seroconversion intervals to those in the neighboring intervals. However, before taking a closer look at the results, the following limitations need to be taken into account:

1) The parents were not instructed to keep a daily diary about the children's symptoms to reduce recall bias. It is likely that the parents failed to recall all of the symptoms, especially those that were mild, after the 3-6 month sampling interval or confused them with the previous interval. Furthermore, of the signs and symptoms discussed here, only lower respiratory tract infections (LRTI) and acute otitis media were always diagnosed by a physician. Other symptoms were more often deduced from the notes of the interviewing study nurse.

2) The statistical power of the analysis is limited by the small number of children involved.

3) The presumed OAS effect may prevent us from correctly timing secondary HBoV infections. Furthermore, secondary infections may not be as strongly associated with symptoms as primary infections due to cross-protective immunity.

With these limitations in mind, Table 11 shows three most commonly observed infection-related symptoms during HBoV1 primary seroconversions and during the neighboring sampling intervals, i.e. upper respiratory tract infection (URTI), gastroenteritis and acute otitis media. URTI was diagnosed when the child's parents reported cough or rhinitis and one or more of the following, sore throat, ear pain or in the case of infants, difficulty breathing or eating. Gastroenteritis was defined as diarrhea, vomiting or both. Acute otitis media was diagnosed by a physician. Other symptoms were observed too rarely for a meaningful assessment of statistical association. The full list of observed symptom types can be found in ref. [139].

Table 11. Infection-related symptoms during primary HBoV1 seroconversions (n=65; children with simultaneous HBoV2- or 3 seroconversion excluded) compared with neighbouring sampling intervals.

Symptom	Interval no. (%)	Next interval no. (%)	p value	Previous interval no. (%)	p value
URTI	30 (46)	20 (31)	0.09	31 (48)	0.86
Acute otitis media	32 (49)	23 (35)	0.15	20 (31)	**0.04**
Gastroenteritis	14 (22)	10 (15)	0.50	11 (17)	0.66

Boldface indicates statistical significance by Liddell exact test (<0.05). URTI, upper respiratory tract illness.

Of the three symptoms shown, only acute otitis media was observed more frequently in the seroconversion interval than in either of the neighboring intervals. However, only comparison of the seroconversion interval to the previous interval yielded a statistically significant difference at the 0.05 probability level. No association was observed between HBoV1 and URTI. This was rather surprising considering that a previous analysis this same cohort of children [139] (done without antibody absorption) showed a strong association between upper respiratory tract illness (URTI) and HBoV1 infection. Potential reasons for this difference are:

1) Smaller number of children classified as having HBoV1 seroconversion. The previous study reported HBoV1 IgG seroconversion in 101 children (the remaining 8 were positive from birth). Study IV found that 15 of these 101 seroconverted children actually had no HBoV1 IgG$_a$ during the entire follow-up period and were instead positive for HBoV2 or -3 IgG$_a$.

2) Better timing of HBoV1 infections in the present study; interval for HBoV1 seroconversion changed in 12 children due to HBoV2-3 cross-reactivity

3) Changes in the interpretation of symptoms fulfilling our criteria of URTI (from the notes of the study nurse)

4) Improved blinding of the statistical data.

It would be tempting to speculate from the frequency of upper respiratory tract symptoms (<50%) in the HBoV1 seroconversion interval that a significant portion of HBoV1 infections does not result in these symptoms. After all, judging from the background symptom rates of the neighboring intervals, other pathogens probably contribute significantly to the symptom rate of 46% in the seroconversion interval. Mostly asymptomatic HBoV1 infections would be in line with most of the existing (DNA-based) literature on the etiology of the virus (chapter 5.2.6). However, the "low" incidence of symptoms in study IV is very likely downplayed by the parents' ability to recall symptomatic episodes and the limitations of the DNA studies have already been discussed extensively. Classification of upper respiratory tract infections is also subjective. Christensen and colleagues, the authors of a study indicating that the detection of respiratory HBoV1 mRNA is mostly limited to symptomatic individuals [98], classified rhinitis and otitis media (alone or in combination) as an upper respiratory tract infection. Our study took a more strict approach required the presence of i) cough or rhinitis together with fever, sore throat and/or ear pain or ii) the simultaneous presence of cough and rhinitis for the symptoms to be classified as an upper respiratory tract infection. It will be interesting to see whether the very high correlation between HBoV1 mRNA detection and symptoms observed by Christensen and colleagues will be replicated by others.

As opposed to URTIs that were reported by the parents and recorded by the study nurse, a physician diagnosed and recorded LRTIs. Of the 65 children with primary (sole) HBoV1 seroconversion, 2 (3%) had LRTI compared to three children (5%) in both of the neighboring intervals. This data indicates that the absolute risk of LRTI in sole HBoV1 infections is quite low. In relative terms, however, the virus can be a significant causal agent in the etiology of LRTI. A recent study by Christensen and colleagues [98] found HBoV1 mRNA in 25% of 133 children with respiratory infections but none of the 28 controls without respiratory symptoms. In fact, 31% of 86 children diagnosed with LRTI tested positive for HBoV1 mRNA and in 19% of the 86 children HBoV1 was the only respiratory virus found.

Turning now to HBoV2, Table 12 illustrates the infection-related symptoms during primary seroconversion by the virus. As expected from the presumed enteric nature of the virus, no association was observed with URTI or acute otitis media. The relative occurrence of gastroenteritis was higher in the seroconversion interval (31%) than in the neighboring intervals (13-18%) or, for

comparison, in the HBoV1 seroconversion interval (22%) without reaching statistical significance. Although the numbers of subjects are small and the data are probably affected by the said recall bias of the parents, it is tempting to speculate that HBoV2 may play some role in gastroenteritis whereas most infections by the virus are asymptomatic or subclinical. This data is in line with previous studies, mostly supportive of an association between HBoV2 DNA detection and acute gastroenteritis [26,93,100].

Table 12. Infection-related symptoms during primary HBoV2 seroconversions compared with neighbouring sampling intervals.

	Interval	Next interval		Previous interval	
Symptom	no. (%)	no. (%)	p value	no. (%)	p value
URTI	14 (31)	18 (18)	0.61	14 (31)	1.0
Acute otitis media	12 (27)	12 (27)	1.0	6 (13)	0.18
Gastroenteritis	14 (31)	8 (18)	0.24	6 (13)	0.08

URTI, upper respiratory tract illness.

9 CONCLUDING REMARKS AND FUTURE PROSPECTS

Recent years have been witnessing the explosion of new viruses being found among humans and animals. The main reason for this is the remarkable innovation in sequencing technologies making random screening of microbial sequences economically feasible. As part of this systematic exploration of novel viruses, at least 14 new parvovirus species have been reported during the last 8 years.

Basic DNA and antibody detection tools are central in assessing the risk that these novel viruses may pose to human health. This thesis describes methods for detecting and quantifying the genomic sequences of all four recently discovered HBoV species. The generation of recombinant HBoV1-4 virus-like particles and their use in the study of HBoV immune responses were also demonstrated.

Future longitudinal studies should address the duration of HBoV2 and HBoV3 shedding in stool to assess the diagnostic value of stool PCR. Future research on the pathogenecity of these enteric viruses could perhaps also adapt the mRNA approach successfully used for the diagnosis of acute HBoV1 infections. Detection of IgA responses is another as yet unexploited avenue in the research of these viruses.

The data on children followed from birth to early teens demonstrated that HBoV1 seroconversion resulted in weak or absent species-specific immune responses against subsequent HBoV2 infections, and vice versa. This may be due to OAS, cross-protective immunity or a combination of both factors. In the absence of animal models for productive HBoV infections, the cross-protective properties of HBoV2-4 antibodies, or lack thereof, could be studied in the recently developed HBoV1 cell culture systems [33,108]. With respect to OAS, preliminary animal experiments by immunizing rabbits consecutively with heterologous or homologous HBoV VLPs have already been done (unpublished). The results were mixed; some immunizations resulted in strong IgG_a responses against the secondary antigen, whereas others resulted (in line with OAS) in very weak secondary responses. It would be interesting to see these experiments replicated with minute doses of antigens to avoid the potential flooding effect discussed in chapter 5.3.2. Live viruses rather than

artificial virus capsids may also be required for OAS, if indeed a real phenomenon, to fully manifest itself.

The DIPP cohort of Study IV could be a rich source of information well beyond what was discussed in this thesis. Analysis of the present data was limited by gaps in the qPCR testing of the serum samples (as selection was based on changes in EIA absorbances) and lack of antibody titer measurements. The omission of these analyses is understandable when one considers the laborious nature of the project; study IV alone represents some ~16 000 EIA and qPCR experiments. Future inclusion of these data would enable to i) address the (unlikely) possibility of non-immunogenic viremias as an alternative to OAS, ii) find more cases viremias among primed individuals, and to iii) identify secondary increases in antibody levels in a more sensitive and objective manner. This would yield a better understanding of the immunological interactions between consecutive HBoV infections.

As we approach the end of this thesis, I cannot help but reflect back on my thoughts about virology during highschool. I thought at the time that a virus would be the perfect subject of biological reaserch for someone who would prefer to understand a system from top to bottom. Something small and simple enough without the uncertainties and massive amount of data involved in the research of more complex organisms. After years of reasearch on one of the smallest and at least structurally most simple viruses known and being overwhelmed by the extent of the literature and our own data, the irony is palbable. To make a circle back to the Carl Sagan quote that prefaced this thesis, we are not at the truth but closer to it, and have found new oceans (or at least reservoirs) of undiscovered possibilities in the process.

10 ACKNOWLEDGEMENTS

The probability of meeting you in the metro
Was the same as randomly picking two particular atoms from a mole of $^{4}_{2}He$
To unite them into $^{8}_{4}Be$
   Almost exactly one in 3.6266191×10^{47}

As you sat there
With your diving equipment
   Epinephrine saturated my cardiac β_1 receptors
   And my iris dilators went into tetanic contraction

You drugged me with dopamine
Seduced me with serotonin
Intoxicated me with oxytocin
Now seeing you after an exhausting day
   Causes complete decompressing
   Downregulates my vasopressin

My love for you has increased
by e^t
where t is the time we have spent together
I wish to shorten my telomers with you
   I wish you feel the same way too

11 SUPPLEMENTARY DATA

Supplementary Table 1. GenBank accession numbers used to construct the phylogenetic tree in Figure 2.

Name	GenBank ID
Adeno-associated virus 2	AAS99314
Aleutian mink disease virus	AAS99314
Bovine parvovirus	NP_041404
California sea lion bocavirus 1	AEM37603
Canine minute virus	ADA57646
Gorilla bocavirus 1	YP_003799998
Human bocavirus 1	YP_338088
Human bocavirus 2	ABW79867
Human bocavirus 3	ACR15791
Human bocavirus 4	YP_002916062
Human partetravirus	ADM26646
Human parvovirus B19	AAB47793.1
Porcine bocavirus 1	ADP36998
Raccoon parvovirus	AAA47117

Supplementary Table 2. GenBank accession numbers used to construct the phylogenetic tree in Supplementary Figure 1.

Name	GenBank ID
Adeno-associated virus 2	AF043303
Aleutian mink disease virus	JN040434
Bovine parvovirus	DQ335247
California sea lion bocavirus 1	JN420361
Canine minute virus	FJ214110
Gorilla bocavirus 1	HM145750
Human bocavirus 1	JQ923422
Human bocavirus 2	FJ973558
Human bocavirus 3	EU918736
Human bocavirus 4	FJ973561
Human partetravirus	AY622943
Human parvovirus B19	AY044266
Porcine bocavirus 1	HM053693
Raccoon parvovirus	JN867610

Supplementary Table 3. GenBank accession numbers and geographical origins of sequences used to construct the DNA alignment for primer and probe design (Figure 16).

Virus	Country of discovery	GenBank ID
HBoV	China	GQ926983
HBoV	China	GU139423
HBoV	Germany	FJ858259
HBoV	Hong Kong	EF450740
HBoV	Sweden	NC_007455
HBoV	Taiwan	EU984233
HBoV	Thailand	EF203920
HBoV	USA	GQ925675
HBoV2	Australia	FJ948860
HBoV2	Australia	EU082213
HBoV2	China	GU301645
HBoV2	China	GU301644
HBoV2	Nigeria	FJ973560
HBoV2	Pakistan	GQ200737
HBoV2	Pakistan	FJ170279
HBoV2	Pakistan	GQ200737
HBoV2	United Kingdom	FJ170280
HBoV3	Australia	EU918736
HBoV3	Australia	FJ948861
HBoV3	Australia	NC_012564
HBoV4	Nigeria	NC_012729
HBoV4	Nigeria	FJ973561
HBoV	China	GQ926983
HBoV	China	GU139423
HBoV	Germany	FJ858259
HBoV	Hong Kong	EF450740
HBoV	Sweden	NC_007455
HBoV	Taiwan	EU984233
HBoV	Thailand	EF203920
HBoV	USA	GQ925675
HBoV2	Australia	FJ948860
HBoV2	Australia	EU082213
HBoV2	China	GU301645
HBoV2	China	GU301644
HBoV2	Nigeria	FJ973560
HBoV2	Pakistan	GQ200737
HBoV2	Pakistan	FJ170279
HBoV2	Pakistan	GQ200737
HBoV2	United Kingdom	FJ170280
HBoV3	Australia	EU918736
HBoV3	Australia	FJ948861
HBoV3	Australia	NC_012564
HBoV4	Nigeria	NC_012729
HBoV4	Nigeria	FJ973561
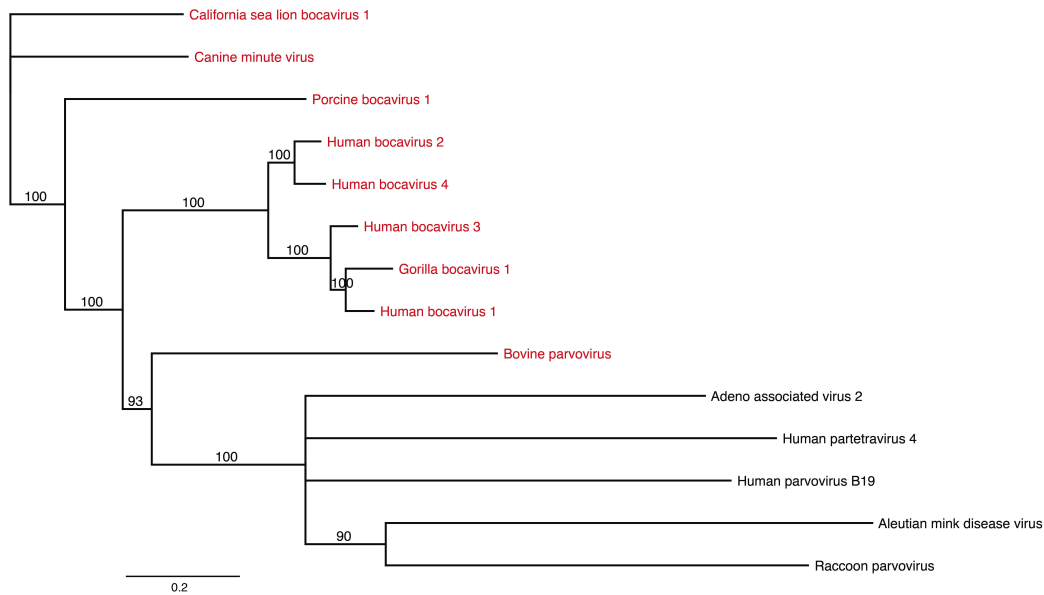
Supplementary Figure 1. Partial neighbor-joining tree of the Parvovirinae subfamily based on the amino acid sequences of the NS1 protein. Genus Bocavirus is shown in red and has been expanded to show most species within the genus, whereas only one exemplary species is shown from the other genera. Sequences were aligned with Geneious 4.8.5 aligner using blosum62 cost matrix with gap open penalty of 12 and gap extension penalty of 3. The tree is derived from 100 bootstrap replicates and bootstrap values ≥70% shown. The scale bar represents 0.2 average amino acid substitutions per site.
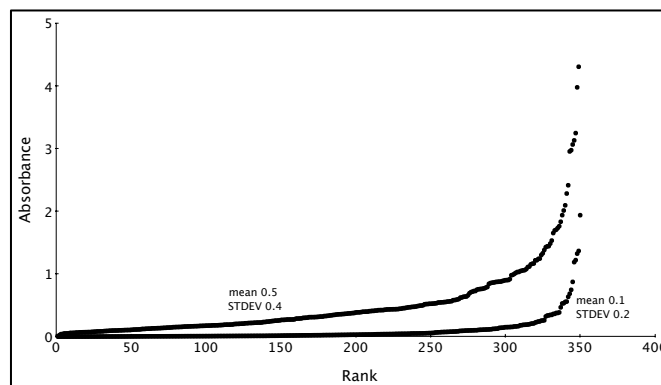
Supplementary Figure 2. Simulation EIA data with 350 lognormally distributed random values. Means and standard deviations (STDEV) of the two different data sets are indicated in the image. The data was generated with the lognrnd function of MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Massachusetts, United States. STDEV, standard deviation.

HBoV1-3 EIA in studies III & IV

All samples

Absorbtion with HBoV2-4 VLPs

Absorption with HBoV1 VLPs

HBoV1 assay → Raw OD

Combined HBoV2-4 assay → Residual OD

Raw OD - residual OD = net HBoV1 OD

HBoV1 assay → Residual OD

HBoV2 assay → Raw OD

HBoV3 assay → Raw OD

Raw OD - residual OD = net HBoV2 or HBoV3 OD

HBoV4 EIA in Study III

All samples

Competition with HBoV1 VLPs

HBoV1 assay → Residual OD

HBoV4 assay → Raw OD

Raw OD - residual OD = net HBoV1 OD

HBoV4 EIA in Study IV

Middlemost and last follow-up sample

Competition with HBoV1-3 VLPs

HBoV4 assay → Raw OD

Combined HBoV1-3 assay → Residual OD

Raw OD - residual OD = net HBoV1 OD

Differentiation betweenHBoV2 and HBoV3 IgG positivity in Study IV

Seroconversion sample and last follow-up sample with HBoV2 and HBoV3 IgG

Competition with HBoV1 + HBoV3 VLPs

HBoV2 assay → Raw OD

Combined HBoV1+HBoV3 assay → Residual OD

Raw OD - residual OD = net HBoV1 OD

Competition with HBoV1 + HBoV2 VLPs

HBoV3 assay → Raw OD

Combined HBoV1+HBoV2 assay → Residual OD

Raw OD - residual OD = net HBoV1 OD
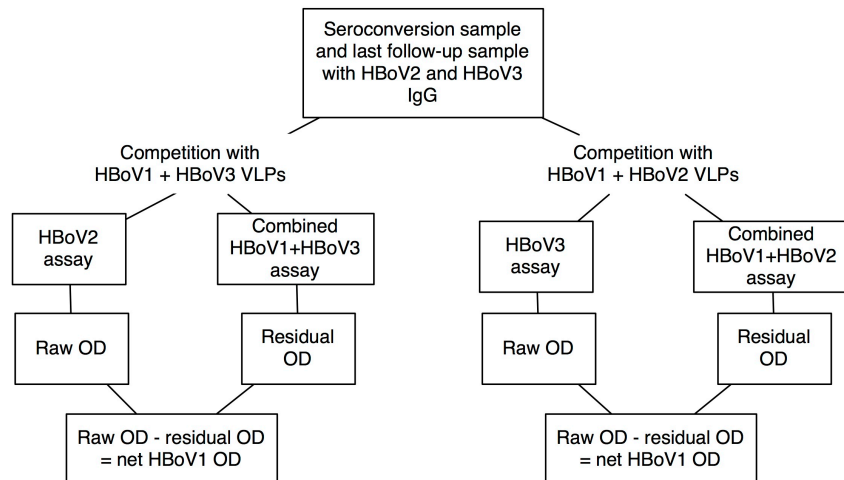
Supplementary Figure 3. Schematic illustration of HBoV2-4 absorption EIA assays. OD, optical density; VLP, virus-like particle.

113

REFERENCES

1. Kaufmann B, Bowman V, Li Y, Szelei J, Waddell P, Tijssen P, Rossmann MG. Structure of Penaeus stylirostris densovirus, a shrimp pathogen. J Virol 2010 1;84:11289–96.

2. Luo M, Tsao J, Rossmann MG, Basak S, Compans RW. Preliminary X-ray crystallographic analysis of canine parvovirus crystals. J Mol Biol 1988;200:209–11.

3. Kerr JR, Cotmore SF, Bloom ME, Linden MR, Parrish C, editors. Parvoviruses. Boca Raton: Hodder Arnold; 2006.

4. Lukashov V, Goudsmit J. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. J Virol 2001 15;75:2729–40.

5. King AM, Adams MJ, Lefkowitz EJ, Carstens EB, editors. Parvoviridae. In: Virus taxonomy: IXth report of the International Committee on Taxonomy of Viruses. London: Academic Press; 2011. p. 405–25.

6. Berns K, Parrish C. Parvoviridae. In: Fields BN, Knipe DM, Howley PM, editors. Fields' virology. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. p. 2437–68.

7. Abinanti FR, Warfield MS. Recovery of a hemadsorbing virus (HADEN) from the gastrointestinal tract of calves. Virology 1961;14:288–9.

8. Binn LN, Lazar EC, Eddy GA, Kajima M. Recovery and characterization of a minute virus of canines. Infect Immun 1970;1:503–8.

9. Kapoor A, Mehta N, Esper F, Poljsak-Prijatelj M, Quan P-L, Qaisar N, Delwart E, Lipkin WI. Identification and characterization of a new bocavirus species in gorillas. PLoS ONE 2010 23;5:e11948.

10. Cheng W-X, Li J-S, Huang C-P, Yao D-P, Liu N, Cui S-X, Jin Y, Duan Z-J. Identification and nearly full-length genome characterization of novel porcine bocaviruses. PLoS ONE 2010 25;5:e13583.

11. Blomström A-L, Belák S, Fossum C, McKillen J, Allan G, Wallgren P, Berg M. Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome. Virus Res 2009;146:125–9.

12. Kapoor A, Mehta N, Dubovi EJ, Simmonds P, Govindasamy L, Medina JL, Street C, Shields S, Lipkin WI. Characterization of novel canine bocaviruses and their association with respiratory disease. J Gen Virol 2012;93:341–6.

13. Li L, Shan T, Wang C, Côte C, Kolman J, Onions D, Gulland F, Delwart E. The fecal viral flora of california sea lions. J Virol 2011 1;85:9909.

14. Carmichael LE, Schlafer DH, Hashimoto A. Minute virus of canines (MVC, canine parvovirus type-1): pathogenicity for pups and seroprevalence estimate. J Vet Diagn Invest 1994;6:165–74.

15. Carmichael LE, Schlafer DH, Hashimoto A. Pathogenicity of minute virus of canines (MVC) for the canine fetus. Cornell Vet 1991;81:151–71.

16. Durham PJ, Lax A, Johnson RH. Pathological and virological studies of experimental parvoviral enteritis in calves. Res Vet Sci 1985;38:209–19.

17. Storz J, Leary JJ, Carlson JH, Bates RC. Parvoviruses associated with diarrhea in calves. J Am Vet Med Assoc 1978;173:624–7.

18. Spahn GJ, Mohanty SB, Hetrick FM. Experimental infection of calves with hemadsorbing enteric (HADEN) virus. Cornell Vet 1966;56:377–86.

19. Pattison JR, Patou G. Parvoviruses. In: S B, editor. Medical microbiology. Galveston: University of Texas; 1996.

20. Ross MH, Pawlina W. Histology: a text and atlas. 6 ed. Philadelphia: Lippincott Williams & Wilkins; 2011.

21. Kauffman SL. Cell proliferation in the mammalian lung. Int Rev Exp Pathol 1980;22:131–91.

22. Atchison R, Casto B, Hammon W. Adenovirus-associated defective virus particles. Science 1965 13;149:754–6.

23. Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. Lancet 1975 11;1:72–3.

24. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. P Natl Acad Sci USA 2005 6;102:12891–6.

25. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. P Natl Acad Sci USA 2001 25;98:11609–14.

26. Arthur JL, Higgins GD, Davidson GP, Givney RC, Ratcliff RM. A novel bocavirus associated with acute gastroenteritis in Australian children. PLoS Pathog 2009;5:e1000391.

27. Kapoor A, Simmonds P, Slikas E, Li L, Bodhidatta L, Sethabutr O, Triki H, Bahri O, Oderinde BS, Baba MM, Bukbuk DN, Besser J, Bartkus J, Delwart E. Human bocaviruses are highly diverse, dispersed, recombination prone, and prevalent in enteric infections. J Infect Dis 2010 1;201:1633–43.

28. Li J, Yang Y, Dong Y, Li Y, Huang Y, Yi Q, Liu K, Li Y. Key elements of

the human bocavirus type 1 (HBoV1) promoter and its trans-activation by NS1 protein. Virol. J. 2013;10:315.

29. Tattersall P, Ward DC. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature 1976 9;263:106–9.

30. Lüsebrink J, Schildgen V, Tillmann RL, Wittleben F, Böhmer A, Müller A, Schildgen O. Detection of head-to-tail DNA sequences of human bocavirus in clinical samples. PLoS ONE 2011;6:e19457.

31. Kapoor A, Hornig M, Asokan A, Williams B, Henriquez JA, Lipkin WI. Bocavirus episome in infected human tissue contains non-identical termini. PLoS ONE 2011 28;6:e21362.

32. Zhao H, Zhao L, Sun Y, Qian Y, Liu L, Jia L, Zhang Y, Dong H. Detection of a bocavirus circular genome in fecal specimens from children with acute diarrhea in Beijing, China. PLoS ONE 2012;7:e48980.

33. Dijkman R, Koekkoek SM, Molenkamp R, Schildgen O, van der Hoek L. Human bocavirus can be cultured in differentiated human airway epithelial cells. J Virol 2009 7;83:7739–48.

34. Chen AY, Cheng F, Lou S, Luo Y, Liu Z, Delwart E, Pintel D, Qiu J. Characterization of the gene expression profile of human bocavirus. Virology 2010 1;403:145–54.

35. Cecchini S, Negrete A, Virag T, Graham BS, Cohen JI, Kotin RM. Evidence of prior exposure to human bocavirus as determined by a retrospective serological study of 404 serum samples from adults in the United States. Clin Vaccine Immunol 2009 4;16:597–604.

36. Bräuniger S, Peters J, Borchers U, Kao M. Further studies on thermal resistance of bovine parvovirus against moist and dry heat. Int J Hyg Environ Health 2000;203:71–5.

37. Gurda BL, Parent KN, Bladek H, Sinkovits RS, DiMattia MA, Rence C, Castro A, McKenna R, Olson N, Brown K, Baker TS, Agbandje-McKenna M. Human bocavirus capsid structure: insights into the structural repertoire of the parvoviridae. J Virol 2010;84:5880–9.

38. Ozawa K, Young N. Characterization of capsid and noncapsid proteins of B19 parvovirus propagated in human erythroid bone marrow cell cultures. J Virol 1987;61:2627–30.

39. Brown CS, Van Lent JW, Vlak JM, Spaan WJ. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J Virol 1991;65:2702–6.

40. Kajigaya S, Fujii H, Field A, Anderson S, Rosenfeld S, Anderson LJ, Shimada T, Young NS. Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. P Natl Acad Sci USA 1991 1;88:4646–50.

41.   Saliki JT, Mizak B, Flore HP, Gettig RR, Burand JP, Carmichael LE, Wood HA, Parrish CR. Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs. J Gen Virol 1992;73 (Pt 2):369–74.

42.   Christensen J, Storgaard T, Bloch B, Alexandersen S, Aasted B. Expression of Aleutian mink disease parvovirus proteins in a baculovirus vector system. J Virol 1993;67:229–38.

43.   Söderlund M, Brown CS, Spaan WJ, Hedman L, Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995;172:1431–6.

44.   Jordan JA. Comparison of a baculovirus-based VP2 enzyme immunoassay (EIA) to an Escherichia coli-based VP1 EIA for detection of human parvovirus B19 immunoglobulin M and immunoglobulin G in sera of pregnant women. J Clin Microbiol 2000;38:1472–5.

45.   Livingston RS, Besselsen DG, Steffen EK, Besch-Williford CL, Franklin CL, Riley LK. Serodiagnosis of mice minute virus and mouse parvovirus infections in mice by enzyme-linked immunosorbent assay with baculovirus-expressed recombinant VP2 Proteins. Clin Vaccine Immunol 2002 1;9:1025–31.

46.   Sharp CP, Lail A, Donfield S, Simmons R, Leen C, Klenerman P, Delwart E, Gomperts ED, Simmonds P. High frequencies of exposure to the novel human parvovirus PARV4 in hemophiliacs and injection drug users, as detected by a serological assay for PARV4 antibodies. J Infect Dis 2009;200:1119–25.

47.   Zádori Z, Szelei J, Lacoste M-C, Li Y, Gariépy S, Raymond P, Allaire M, Nabi IR, Tijssen P. A viral phospholipase A2 is required for parvovirus infectivity. Dev Cell 2001;1:291–302.

48.   Farr GA. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. P Natl Acad Sci USA 2005 22;102:17148–53.

49.   Vihinen-Ranta M, Wang D, Weichert WS, Parrish CR. The VP1 N-terminal sequence of canine parvovirus affects nuclear transport of capsids and efficient cell infection. J Virol 2002;76:1884–91.

50.   Bönsch C, Zuercher C, Lieby P, Kempf C, Ros C. The globoside receptor triggers structural changes in the B19 virus capsid that facilitate virus internalization. J Virol 2010;84:11737–46.

51.   Jartti T, Hedman K, Jartti L, Ruuskanen O, Allander T, Söderlund-Venermo M. Human bocavirus-the first 5 years. Rev Med Virol 2012;22:46–64.

52.   Fry AM, Lu X, Chittaganpitch M, Peret T, Fischer J, Dowell SF, Anderson LJ, Erdman D, Olsen SJ. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis 2007 1;195:1038–45.

53.   Han T-H, Chung J-Y, Hwang E-S. Human bocavirus 2 in children, South Korea. Emerg Infect Dis 2009 1;15:1698–700.

54.   Han T-H, Chung J-Y, Koo JW, Kim SW, Hwang E-S. WU polyomavirus in children with acute lower respiratory tract infections, South Korea. Emerg Infect Dis 2007 1;13:1766.

55.   Longtin J, Bastien M, Gilca R, Leblanc E, de Serres G, Bergeron M, Boivin G. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis 2008 1;14:217.

56.   Allander T, Jartti T, Gupta S, Niesters HGM, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypiä T, Ruuskanen O. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007 1;44:904–10.

57.   Martin ET, Fairchok MP, Kuypers J, Magaret A, Zerr DM, Wald A, Englund JA. Frequent and prolonged shedding of bocavirus in young children attending daycare. J Infect Dis 2010 1;201:1625–32.

58.   Weissbrich B, Neske F, Schubert J, Tollmann F, Blath K, Blessing K, Kreth HW. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis 2006;6:109.

59.   Yoshida LM, Suzuki M, Yamamoto T, Nguyen HA, Nguyen CD, Nguyen AT, Oishi K, Vu TD, Le TH, Le MQ, Yanai H, Kilgore PE, Dang DA, Ariyoshi K. Viral pathogens associated with acute respiratory infections in central vietnamese children. Pediatr Infect Dis J 2010;29:75–7.

60.   Foulongne V, Olejnik Y, Perez V, Elaerts S, Rodière M, Segondy M. Human bocavirus in French children. Emerg Infect Dis 2006;12:1251–3.

61.   Bastien N, Brandt K, Dust K, Ward D, Li Y. Human Bocavirus infection, Canada. Emerg Infect Dis 2006;12:848–50.

62.   Calvo C, Pozo F, García-García ML, Sanchez M, Lopez-Valero M, Pérez-Breña P, Casas I. Detection of new respiratory viruses in hospitalized infants with bronchiolitis: a three-year prospective study. Acta Paediatr 2010 16;99:883–7.

63.   Regamey N, Frey U, Deffernez C, Latzin P, Kaiser L. Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatr Infect Dis J 2007;26:177–9.

64.   Miron D, Srugo I, Kra-Oz Z, Keness Y, Wolf D, Amirav I, Kassis I. Sole pathogen in acute bronchiolitis: is there a role for other organisms apart from respiratory syncytial virus? Pediatr Infect Dis J 2010;29:e7–e10.

65.   Linstow von M-L, Høgh M, Høgh B. Clinical and epidemiologic characteristics of human bocavirus in Danish Infants. Pediatr Infect Dis J 2008;27:897–902.

66.   Lu X, Chittaganpitch M, Olsen SJ, Mackay IM, Sloots TP, Fry AM, Erdman DD. Real-time PCR assays for detection of bocavirus in human

specimens. J Clin Microbiol 2006 1;44:3231–5.

67.   Chieochansin T, Kapoor A, Delwart E, Poovorawan Y, Simmonds P. Absence of detectable replication of human bocavirus species 2 in respiratory tract. Emerg Infect Dis 2009;15:1503–5.

68.   Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, Kahn JS. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 2006 1;194:1276–82.

69.   Brieu N, Guyon G, Rodière M, Segondy M, Foulongne V. Human bocavirus infection in children with respiratory tract disease. Pediatr Infect Dis J 2008;27:969–73.

70.   Christensen A, Nordbø SA, Krokstad S, Rognlien AGW, Døllner H. Human bocavirus in children: mono-detection, high viral load and viraemia are associated with respiratory tract infection. J Clin Virol 2010;49:158–62.

71.   Lu X, Gooding LR, Erdman DD. Human bocavirus in tonsillar lymphocytes. Emerg Infect Dis 2008;14:1332–4.

72.   Lehtoranta L, Söderlund-Venermo M, Nokso-Koivisto J, Toivola H, Blomgren K, Hatakka K, Poussa T, Korpela R, Pitkäranta A. Human bocavirus in the nasopharynx of otitis-prone children. Int J Pediatr Otorhinolaryngol 2012;76:206–11.

73.   Blessing K, Neske F, Herre U, Kreth H-W, Weissbrich B. Prolonged detection of human bocavirus DNA in nasopharyngeal aspirates of children with respiratory tract disease. Pediatr Infect Dis J 2009;28:1018–9.

74.   Chan KH, Peiris JSM, Lim W, Nicholls JM, Chiu SS. Comparison of nasopharyngeal flocked swabs and aspirates for rapid diagnosis of respiratory viruses in children. J Clin Virol 2008;42:65–9.

75.   Heikkinen T, Marttila J, Salmi AA, Ruuskanen O. Nasal swab versus nasopharyngeal aspirate for isolation of respiratory viruses. J Clin Microbiol 2002;40:4337–9.

76.   Zhao B, Yu X, Wang C, Teng Z, Wang C, Shen J, Gao Y, Zhu Z, Wang J, Yuan Z, Wu F, Zhang X, Ghildyal R. High human bocavirus viral load is associated with disease severity in children under five years of age. PLoS ONE 2013;8:e62318.

77.   Martin ET, Taylor J, Kuypers J, Magaret A, Wald A, Zerr D, Englund JA. Detection of bocavirus in saliva of children with and without respiratory illness. J Clin Microbiol 2009 30;47:4131–2.

78.   García-García ML, Calvo C, Pozo F, Pérez-Breña P, Quevedo S, Bracamonte T, Casas I. Human bocavirus detection in nasopharyngeal aspirates of children without clinical symptoms of respiratory infection. Pediatr Infect Dis J 2008;27:358–60.

References

79.   Cheng W-X, Jin Y, Duan Z-J, Xu Z-Q, Qi H-M, Zhang Q, Yu J-M, Zhu L, Jin M, Liu N, Cui S-X, Li H-Y, Fang Z-Y. Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clin Infect Dis 2008 15;47:161–7.

80.   Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol 2007;38:321–5.

81.   Norja P, Hokynar K, Aaltonen L-M, Chen R, Ranki A, Partio EK, Kiviluoto O, Davidkin I, Leivo T, Eis-Hübinger AM, Schneider B, Fischer H-P, Tolba R, Vapalahti O, Vaheri A, Söderlund-Venermo M, Hedman K. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. P Natl Acad Sci USA 2006 9;103:7450–3.

82.   Söderlund M, Essen von R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet 1997 12;349:1063–5.

83.   Kuethe F, Lindner J, Matschke K, Wenzel JJ, Norja P, Ploetze K, Schaal S, Kamvissi V, Bornstein SR, Schwanebeck U, Modrow S. Prevalence of parvovirus B19 and human bocavirus DNA in the heart of patients with no evidence of dilated cardiomyopathy or myocarditis. Clin Infect Dis 2009 1;49:1660–6.

84.   Norja P, Hedman L, Kantola K, Kemppainen K, Suvilehto J, Pitkäranta A, Aaltonen L-M, Seppänen M, Hedman K, Söderlund-Venermo M. Occurrence of human bocaviruses and parvovirus 4 in solid tissues. J Med Virol 2012 18;84:1267–73.

85.   Schildgen V, Malecki M, Tillmann RL, Brockmann M, Schildgen O. The human bocavirus is associated with some lung and colorectal cancers and persists in solid tumors. PLoS ONE 2013 27;8:e68020.

86.   Pozo F, García-García ML, Calvo C, Cuesta I, Pérez-Breña P, Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol 2007;40:224–8.

87.   Nakanishi K, Tsugawa T, Honma S, Nakata S, Tatsumi M, Yoto Y, Tsutsumi H. Detection of enteric viruses in rectal swabs from children with acute gastroenteritis attending the pediatric outpatient clinics in Sapporo, Japan. J Clin Virol 2009;46:94–7.

88.   Lau SKP, Yip CCY, Que TL, Lee RA, Au Yeung RKH, Zhou B, So LY, Lau YL, Chan KH, Woo PCY, Yuen KY. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis 2007;196:986–93.

89.   Vicente D, Cilla G, Montes M, Pérez-Yarza EG, Pérez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 2007 1;13:636–7.

90.   Lee JI, Chung J-Y, Han T-H, Song M-O, Hwang E-S. Detection of

human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis 2007 1;196:994–7.

91. Yu J-M, Li D-D, Xu Z-Q, Cheng W-X, Zhang Q, Li H-Y, Cui S-X, Miao-Jin, Yang S-H, Fang Z-Y, Duan Z-J. Human bocavirus infection in children hospitalized with acute gastroenteritis in China. J Clin Virol 2008;42:280–5.

92. Proenca-Jodena J, Martinez M, Amarilla AA, Espínola EE, Galeano ME, Farina N, Russomando G, Aquino VH, Parra GI, Arruda E. Viral load of human bocavirus-1 in stools from children with viral diarrhoea in Paraguay. Epidemiol Infect 2013 21;:1–5.

93. Nawaz S, Allen DJ, Aladin F, Gallimore C, Iturriza-Gómara M. Human bocaviruses are not significantly associated with gastroenteritis: results of retesting archive DNA from a case control study in the UK. PLoS ONE 2012;7:e41346.

94. Enders M, Lindner J, Wenzel JJ, Baisch C, Schalasta G, Enders G, Modrow S. No detection of human bocavirus in amniotic fluid samples from fetuses with hydrops or isolated effusions. J Clin Virol 2009;45:300–3.

95. Riipinen A, Väisänen E, Lahtinen A, Karikoski R, Nuutila M, Surcel H-M, Taskinen H, Hedman K, Söderlund-Venermo M. Absence of human bocavirus from deceased fetuses and their mothers. J Clin Virol 2010;47:186–8.

96. Song J-R, Jin Y, Xie Z-P, Gao H-C, Xiao N-G, Chen W-X, Xu Z-Q, Yan K-L, Zhao Y, Hou Y-D, Duan Z-J. Novel human bocavirus in children with acute respiratory tract infection. Emerg Infect Dis 2010 1;16:324–7.

97. Koseki N, Teramoto S, Kaiho M, Gomi-Endo R, Yoshioka M, Takahashi Y, Nakayama T, Sawada H, Konno M, Ushijima H, Kikuta H, Ariga T, Ishiguro N. Detection of human bocaviruses 1 to 4 from nasopharyngeal swab samples collected from patients with respiratory tract infections. J Clin Microbiol 2012;50:2118–21.

98. Christensen A, Døllner H, Skanke LH, Krokstad S, Moe N, Nordbø SA. Detection of spliced mRNA from human bocavirus 1 in clinical samples from children with respiratory tract infections. Emerg Infect Dis 2013;19:574–80.

99. Han T-H, Kim C-H, Park S-H, Kim E-J, Chung J-Y, Hwang E-S. Detection of human bocavirus-2 in children with acute gastroenteritis in South Korea. Arch Virol 2009 28;154:1923–7.

100. Jin Y, Cheng W-X, Xu Z-Q, Liu N, Yu J-M, Li H-Y, Jin M, Li D-D, Zhang Q, Duan Z-J. High prevalence of human bocavirus 2 and its role in childhood acute gastroenteritis in China. J Clin Virol 2011;52:251–3.

101. Xu Z-Q, Cheng W-X, Li B-W, Li J, Lan B, Duan Z-J. Development of a real-time PCR assay for detecting and quantifying human bocavirus 2. J Clin Microbiol 2011;49:1537–41.

102.   Risku M, Kätkä M, Lappalainen S, Räsänen S, Vesikari T. Human
        bocavirus types 1, 2 and 3 in acute gastroenteritis of childhood. Acta
        Paediatr. 2012;101:e405–10.

103.   Kapoor A, Slikas E, Simmonds P, Chieochansin T, Naeem A, Shaukat S,
        Alam M, Sharif S, Angez M, Zaidi S, Delwart E. A newly identified
        bocavirus species in human stool. J Infect Dis 2008 15;

104.   Chow BDW, Ou Z, Esper FP. Newly recognized bocaviruses (HBoV,
        HBoV2) in children and adults with gastrointestinal illness in the
        United States. J Clin Virol 2010;47:143–7.

105.   Blinkova O, Rosario K, Li L, Kapoor A, Slikas B, Bernardin F, Breitbart
        M, Delwart E. Frequent detection of highly diverse variants of
        cardiovirus, cosavirus, bocavirus, and circovirus in sewage samples
        collected in the United States. J Clin Microbiol 2009 1;47:3507.

106.   Wang Y, Gonzalez R, Zhou H, Li J, Li Y, Paranhos-Baccalà G, Vernet G,
        Guo L, Wang J. Detection of human bocavirus 3 in China. Eur J Clin
        Microbiol Infect Dis 2011 1;30:799–805.

107.   Fredericks DN, Relman DA. Sequence-based identification of microbial
        pathogens: a reconsideration of Koch's postulates. Clin Micriobiol Rev
        1996;9:18–33.

108.   Huang Q, Deng X, Yan Z, Cheng F, Luo Y, Shen W, Lei-Butters DCM,
        Chen AY, Li Y, Tang L, Söderlund-Venermo M, Engelhardt JF, Qiu J.
        Establishment of a reverse genetics system for studying human
        bocavirus in human airway epithelia. PLoS Pathog 2012;8:e1002899.

109.   Rommelaere J, Cornelis JJ. Antineoplastic activity of parvoviruses. J
        Virol Methods 1991;33:233–51.

110.   Jula A, Waris M, Kantola K, Peltola V, Söderlund-Venermo M, Hedman
        K, Ruuskanen O. Primary and secondary human bocavirus 1 infections
        in a family, Finland. Emerg Infect Dis 2013;19:1328–31.

111.   Edner N, Castillo-Rodas P, Falk L, Hedman K, Söderlund-Venermo M,
        Allander T. Life-threatening respiratory tract disease with human
        bocavirus-1 infection in a 4-year-old child. J Clin Microbiol 2012
        20;50:531–2.

112.   Ursic T, Steyer A, Kopriva S, Kalan G, Krivec U, Petrovec M. Human
        bocavirus as the cause of a life-threatening infection. J Clin Microbiol
        2011;49:1179–81.

113.   Söderlund-Venermo M, Lahtinen A, Jartti T, Hedman L, Kemppainen
        K, Lehtinen P, Allander T, Ruuskanen O, Hedman K. Clinical
        assessment and improved diagnosis of bocavirus-induced wheezing in
        children, Finland. Emerg Infect Dis 2009;15:1423–30.

114.   Fuller SD, Bonsdorff von CH, Simons K. Cell surface influenza
        haemagglutinin can mediate infection by other animal viruses. EMBO J
        1985;4:2475–85.

115. Chi B, Dickensheets HL, Spann KM, Alston MA, Luongo C, Dumoutier L, Huang J, Renauld J-C, Kotenko SV, Roederer M, Beeler JA, Donnelly RP, Collins PL, Rabin RL. Alpha and lambda interferon together mediate suppression of CD4 T cells induced by respiratory syncytial virus. J Virol 2006;80:5032–40.

116. Morens DM, Burke DS, Halstead SB. The wages of original antigenic sin. Emerg Infect Dis 2010 1;16:1023–4.

117. Davenport FM, Jensen PN, Hennessy AV, Francis T. Characterization of influenza antibodies by serum absorption. J Exp Med 1956 1;104:199–209.

118. Davenport FM, Hennessy AV. A serologic recapitulation of past experiences with influenza A; antibody response to monovalent vaccine. J Exp Med 1956 1;104:85–97.

119. Francis T. On the doctrine of original antigenic sin. P Am Philos Soc 1960;104:572–8.

120. Amesh A Adalja DAH. Original Antigenic Sin and Pandemic (H1N1) 2009. Emerg Infect Dis 2010 1;16:1028.

121. Virelizier JL, Allison AC, Schild GC. Antibody responses to antigenic determinants of influenza virus hemagglutinin. II. Original antigenic sin: a bone marrow-derived lymphocyte memory phenomenon modulated by thymus-derived lymphocytes. J Exp Med 1974 1;140:1571–8.

122. Halstead SB, Rojanasuphot S, Sangkawibha N. Original antigenic sin in dengue. Am J Trop Med Hyg 1983;32:154–6.

123. Inouye S, Matsuno S, Tsurukubo Y. "Original antigenic sin" phenomenon in experimental flavivirus infections of guinea pigs: studies by enzyme-linked immunosorbent assay. Microbiol Immunol 1984;28:569–74.

124. Chan PL, Sinclair NRS. Regulation of the immune response: V. An analysis of the function of the Fc portion of antibody in suppression of an immune response with respect to interaction with components of the lymphoid system. Immunology 1971 1;21:967.

125. Phillips NE, Parker DC. Cross-linking of B lymphocyte Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. J Immunol 1984;132:627–32.

126. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. Nature 1994;369:340.

127. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng N-Y, Mays I, Garman L, Helms C, James J, Air GM, Capra JD, Ahmed R, Wilson PC. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature 2008 29;453:667–71.

128.   Kim JH, Skountzou I, Compans R, Jacob J. Original antigenic sin responses to influenza viruses. J Immunol 2009 1;183:3294–301.

129.   Hawkes RA. Enhancement of the infectivity of arboviruses by specific antisera produced in domestic fowls. Aust J Exp Biol Med Sci 1964;42:465–82.

130.   Kanno H, Wolfinbarger JB, Bloom ME. Aleutian mink disease parvovirus infection of mink macrophages and human macrophage cell line U937: demonstration of antibody-dependent enhancement of infection. J Virol 1993;67:7017–24.

131.   Tamura M, Webster RG, Ennis FA. Subtype cross-reactive, infection-enhancing antibody responses to influenza A viruses. J Virol 1994;68:3499–504.

132.   Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol 2007;5:518–28.

133.   Bravo JR, Guzman MG, Kouri GP. Why dengue haemorrhagic fever in Cuba? 1. Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Trans R Soc Trop Med Hyg 1987;81:816–20.

134.   Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 2010;6:e1000790.

135.   Antikainen J, Tarkka E, Haukka K, Siitonen A, Vaara M, Kirveskari J. New 16-plex PCR method for rapid detection of diarrheagenic Escherichia coli directly from stool samples. Eur J Clin Microbiol Infect Dis 2009 24;28:899–908.

136.   Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus ADME, Ruuskanen O. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis 2004;10:1095–101.

137.   Hedman L, Söderlund-Venermo M, Jartti T, Ruuskanen O, Hedman K. Dating of human bocavirus infection with protein-denaturing IgG-avidity assays-Secondary immune activations are ubiquitous in immunocompetent adults. J Clin Virol 2010;48:44–8.

138.   Ilonen J, Sjöroos M, Knip M, Veijola R, Simell O, Akerblom HK, Paschou P, Bozas E, Havarani B, Malamitsi-Puchner A, Thymelli J, Vazeou A, Bartsocas CS. Estimation of genetic risk for type 1 diabetes. Am J Med Genet 2002 30;115:30–6.

139.   Meriluoto M, Hedman L, Tanner L, Simell V, Mäkinen M, Simell S, Mykkänen J, Korpelainen J, Ruuskanen O, Ilonen J, Knip M, Simell O, Hedman K, Söderlund-Venermo M. Association of human bocavirus 1 infection with respiratory disease in childhood follow-up study, Finland. Emerg Infect Dis 2012;18:264–71.

140.   Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003 1;31:3406–15.

141.   Chang MJ, Kuzio J, Blissard GW. Modulation of translational efficiency by contextual nucleotides flanking a baculovirus initiator AUG codon. Virology 1999 5;259:369–83.

142.   Peakman TC, Charles IG, Sydenham MA, Gewert DR, Page MJ, Makoff AJ. Enhanced expression of recombinant proteins in insect cells using a baculovirus vector containing a bacterial leader sequence. Nucleic Acids Res 1992 25;20:6111–2.

143.   Schwab C, Bosshard HR. Caveats for the use of surface-adsorbed protein antigen to test the specificity of antibodies. J Immunol Methods 1992 14;147:125–34.

144.   Kean JM, Rao S, Wang M, Garcea RL. Seroepidemiology of human polyomaviruses. PLoS Pathog 2009 1;5:e1000363.

145.   Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009 6;55:611–22.

146.   Söderlund M, Brown KE, Meurman O, Hedman K. Prokaryotic expression of a VP1 polypeptide antigen for diagnosis by a human parvovirus B19 antibody enzyme immunoassay. J Clin Microbiol 1992 1;30:305–11.

147.   Ros C, Gerber M, Kempf C. Conformational changes in the VP1-unique region of native human parvovirus B19 lead to exposure of internal sequences that play a role in virus neutralization and infectivity. J Virol 2006;80:12017–24.

148.   Lindner J, Zehentmeier S, Franssila R, Barabas S, Schroeder J, Deml L, Modrow S. CD4+ T helper cell responses against human bocavirus viral protein 2 viruslike particles in healthy adults. J Infect Dis 2008 1;198:1677–84.

149.   Kaikkonen L, Lankinen H, Harjunpää I, Hokynar K, Söderlund-Venermo M, Oker-Blom C, Hedman L, Hedman K. Acute-phase-specific heptapeptide epitope for diagnosis of parvovirus B19 infection. J Clin Microbiol 1999;37:3952–6.

150.   Guo L, Wang Y, Zhou H, Wu C, Song J, Li J, Paranhos-Baccalà G, Vernet G, Wang J, Hung T. Differential seroprevalence of human bocavirus species 1-4 in Beijing, China. PLoS ONE 2012;7:e39644.

151.   Esser C, Radbruch A. Immunoglobulin class switching: molecular and cellular analysis. Annu Rev Immunol 1990;8:717–35.

152.   Midgley CM, Bajwa-Joseph M, Vasanawathana S, Limpitikul W, Wills B, Flanagan A, Waiyaiya E, Tran HB, Cowper AE, Chotiyarnwon P, Grimes JM, Yoksan S, Malasit P, Simmons CP, Mongkolsapaya J, Screaton GR. An in-depth analysis of original antigenic sin in dengue

virus infection. J Virol 2011;85:410–21.

153. van Panhuis WG, Gibbons RV, Endy TP, Rothman AL, Srikiatkhachorn A, Nisalak A, Burke DS, Cummings DAT. Inferring the serotype associated with dengue virus infections on the basis of pre- and postinfection neutralizing antibody titers. J Infect Dis 2010 1;202:1002–10.

154. Endo R, Ishiguro N, Kikuta H, Teramoto S, Shirkoohi R, Ma X, Ebihara T, Ishiko H, Ariga T. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. J Clin Microbiol 2007;45:3218–23.

155. Kahn JS, Kesebir D, Cotmore SF, D'abramo A Jr, Cosby C, Weibel C, Tattersall P. Seroepidemiology of Human Bocavirus Defined Using Recombinant Virus-Like Particles. J Infect Dis 2008 1;198:41–50.

156. Lindner J, Karalar L, Zehentmeier S, Plentz A, Pfister H, Struff W, Kertai M, Segerer H, Modrow S. Humoral immune response against human bocavirus VP2 virus-like particles. Viral Immunol 2008;21:443–9.

157. Guido M, Zizza A, Bredl S, Lindner J, De Donno A, Quattrocchi M, Grima P, Modrow S, Seroepidemiology Group. Seroepidemiology of human bocavirus in Apulia, Italy. Clin Microbiol Infect 2012;18:E74–6.

158. Karalar L, Lindner J, Schimanski S, Kertai M, Segerer H, Modrow S. Prevalence and clinical aspects of human bocavirus infection in children. Clin Microbiol Infect 2010;16:633–9.

159. Bonvicini F, Manaresi E, Gentilomi GA, Di Furio F, Zerbini M, Musiani M, Gallinella G. Evidence of human bocavirus viremia in healthy blood donors. Diagn Microbiol Infect Dis 2011;71:460–2.

160. Musiani M, Zerbini M, Gentilomi G, Plazzi M, Gallinella G, Venturoli S. Parvovirus B19 clearance from peripheral blood after acute infection. J Infect Dis 1995;172:1360–3.

161. Lindblom A, Isa A, Norbeck O, Wolf S, Johansson B, Broliden K, Tolfvenstam T. Slow clearance of human parvovirus B19 viremia following acute infection. Clin Infect Dis 2005 15;41:1201–3.

162. Mitui MT, Tabib SMSB, Matsumoto T, Khanam W, Ahmed S, Mori D, Akhter N, Yamada K, Kabir L, Nishizono A, Söderlund-Venermo M, Ahmed K. Detection of human bocavirus in the cerebrospinal fluid of children with encephalitis. Clin Infect Dis 2012;54:964–7.

163. Paloniemi M, Lappalainen S, Salminen M, Kätkä M, Kantola K, Hedman L, Hedman K, Söderlund-Venermo M, Vesikari T. Human bocaviruses are commonly found in stools of hospitalized children without causal association to acute gastroenteritis. Eur J Pediatr 2014 4. In print. DOI 10.1007/s00431-014-2290-x.